alkylamine thereon suitable for immobilizing or fixing any negatively charged polyelectrolytes applied thereto."

Thus the exposed amines form non-covalent bonds with random negative phosphate groups on a nucleic acid's backbone. The bonding in this situation is neither covalent nor by a terminal nucleotide, and so the examiner's interpretation of Stavrianopoulos is incorrect.

The Applicant previously referred the examiner to the first page of Dawson *et al.*, 2005, teaching that "Noncovalent immobilization (including the use of polylysine-coated and aminopropylsilane-coated slides) is commonly used for gene expression microarrays ...".

The glass derivatisation chemistry used and disclosed in Stavrianopoulos gives a charged surface that retains DNA by non-covalent means *e.g.* ionic interactions between a positive surface and negatively-charged phosphates on a nucleic acid backbone.

The charged nature of the derivatised surface is further confirmed by the following references, copies of which are enclosed.

- Carre et al. *J Colloid Interface Sci.* 2003 Apr 1;260(1):49-55, teaches that "The objective of this work is to model simply the interface interactions between DNA and glass slides modified with an aminosilane (gamma-aminopropyltriethoxysilane, APTS) ... A glass substrate treated with APTS is positively or negatively charged, depending on the pH... This provides an indication of the role of ionic interactions in the adsorption of DNA molecules onto aminated glass slides." (emphasis added). See the abstract.

- Satou et al. *J Gen Microbiol.* 1988 May; 134(5):1299-305, teaches that "Aminopropyl glass was prepared by alkylsilylation of glass slides with gamma-aminopropyltriethoxysilane. This glass carries primary amino groups which may be protonated at pH 7.2." (emphasis added). See the abstract. This paper does not relate to DNA attachment but does describe that APTS reacts with glass to give an ionisable surface. This reinforces what Stavrianopoulos already says at col. 8, lines 32ff, about retention of negatively-charged substances.

- Simiarly, Feilner et al. *Current Proteomics* 2004 1:283-95, teaches that "Surfaces, which allow non-covalent attachment, can be positively charged (e.g. poly-L-

lysine, aminosilane) or hydrophobic (e.g. nitrocellulose, polystyrene).". See page 284, left column, last complete paragraph. The focus is on retaining protein but, again, it is clear that the nature of the surface is for ionic interaction.

- Levina et al. *Biotechnol J.* 2007 Jul;2(7):879-85, teaches that "Various materials, such as glass, plastic, metals, etc., are utilized for preparing DNA chips. In each particular case special approaches are used for immobilization of different oligonucleotide derivatives on the solid supports. We describe a general technique for DNA chips preparation on various unmodified surfaces using one type of oligonucleotide derivative, polylysine-oligonucleotide conjugates (PL-oligo). A long polyamine spacer in the PL-oligo conjugates provides a durable irreversible non-covalent immobilization ..." (emphasis added). See the abstract.

In view of the foregoing, it is respectfully submitted that the glass derivatisation chemistry used and disclosed in Stavrianopoulos gives a charged surface that retains DNA by non-covalent means *e.g.* ionic interactions between a positive surface and negatively-charged phosphates on a nucleic acid backbone.

Favorable action and allowance is solicited.

Respectfully submitted,

Edwin Southern

By: 

Warren M. Cheek, Jr.
Registration No. 33,367
Attorney for Applicant

WMC/dlk
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
December 14, 2007.

ACADEMIC PRESS

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Journal of Colloid and Interface Science 260 (2003) 49–55

JOURNAL OF
Colloid and
Interface Science

www.elsevier.com/locate/jcis

Molecular interactions between DNA and an aminated glass substrate

Alain Carré,* Valérie Lacarrière, and William Birch

Corning S.A., Fontainebleau Research Center, 7 bis, Avenue de Valvins, 77210 Avon, France

Received 3 July 2002; accepted 7 November 2002

Abstract

With the development of DNA arrays, the immobilization of DNA strands onto solid substrates remains an essential research topic. DNA arrays have potential applications in DNA sequencing, mutation detection, and pathogen identification. DNA bound to solid substrates must still be accessible and retain the ability to hybridize with its complementary strands. One technology to produce these arrays involves linking DNA molecule probes to a silanized substrate in microspot patterns and exposing them to a solution of fluorescently labeled samples of DNA targets. The behavior of both the target and probe DNA and their interactions with each other at the substrate surface, particularly with respect to molecular interactions, are poorly understood at the present time. The objective of this work is to model simply the interface interactions between DNA and glass slides modified with an aminosilane (γ-aminopropyltriethoxysilane, APTS). In aqueous solutions, DNA behaves as a polyacid over a wide range of pH. A glass substrate treated with APTS is positively or negatively charged, depending on the pH. A model of the surface charge of APTS-treated glass has been developed from results of wetting experiments performed at various pH. It has been demonstrated that the surface charge of APTS-treated glass is well described by a model of constant capacitance of the electrical double layer. A good correlation between experimental data on DNA retention at various pH's and the variation of the surface charge of the APTS-treated glass is obtained. This provides an indication of the role of ionic interactions in the adsorption of DNA molecules onto aminated glass slides.

Keywords: DNA; Glass; Aminosilane; Aminopropyltriethoxysilane; Surface charge; Point of zero charge; Ionic interaction; DNA retention; Wettability; Contact angle; Water; pH

1. Introduction

Over the past years, nucleic acid probes have been extensively used to detect human pathogens in molecular recognition tests. With the development of DNA microarrays, the immobilization of these molecules onto solid substrates remains an essential research topic. When bound to a solid substrate, the DNA molecules must still be accessible and retain the ability to hybridize with their complementary strands. Hybridization of the fluorescently labeled probe to a specific DNA sequence generally indicates that the gene represented by this sequence is expressed. DNA arrays have potential applications in DNA sequencing, mutation detection, and pathogen identification. Preparation of DNA arrays involves binding of DNA molecules to silanized glass substrates in the shape of microspots, or synthesizing DNA on the substrate and exposing the DNA array to a solution of fluorescently labeled samples of DNA targets. This process, which is sometimes called heterogeneous hybridization, allows conducting many different tests in parallel, using very small amounts of expensive DNA probe material. However, the behavior of both the target and probe DNA and their interaction with each other at the liquid/substrate interface are at the present time poorly understood, particularly with respect to specific and nonspecific molecular interactions.

Modified and bare silica or glass substrates are currently used in many fields of technology to immobilize genetic material. Bare silica has been found to be reactive but prone to rapid contamination. Modification of silica or glass surfaces by covalent binding of silanes with surface silanol groups has been extensively studied. Silanization of glass substrates with an aminosilane provides an amine-rich surface which is ideal for attachment of DNA to the solid substrate.

It is generally known that both electrostatic and hydrophobic interactions are major driving forces for protein adsorption at solid/liquid interfaces. However, relatively little is known about how these interactions affect the interfacial behavior of single- or double-stranded DNA molecules.

* Corresponding author.
E-mail address: carrea@corning.com (A. Carré).

0021-9797/03/$ – see front matter
doi:10.1016/S0021-9797(02)00147-9

It has been demonstrated that DNA oligonucleotides in solution interact with aminopropyl silane treated substrates via ionic interactions [1]. The pH of the medium in which oligonucleotides are present has a strong impact on the amount of material adsorbed and retained on aminated substrates [2].

The objective of this work is to model simply the interaction between DNA molecules and an aminated glass slide as a function of the pH and to use experimental techniques to support the model. The surface charge of the substrate will be studied with the evolution of the wettability of the aminated glass as a function of the pH of the contacting water. The variation of the DNA retention on the substrate as a function of pH will be quantified using fluorescent detection.

2. Theory

2.1. Charge of DNA in solution

DNA is a polymer with an alternating sugar–phosphate sequence. The monomer units of DNA are nucleotides. Each nucleotide consists of a deoxyribose sugar, a nitrogen-containing base attached to the sugar, and a phosphate group. There are four different types of nucleotides found in DNA, differing only in the nitrogenous base: adenine, guanine, cytosine, and thymine.

Native DNA is a double-stranded structure that may be denatured into two single strands in a chaotropic solvent or at high pH values or by heating (melting). The denaturation is a reversible process.

When a single-stranded DNA molecule is in water, ionization of sites on the linkages and bases results in a charge that is pH-dependent. The electrostatic characteristics of a single-stranded DNA molecule are derived from the ionization of the phosphodiester linkages, resulting in one negative charge per nucleotide, and from ionization of bases, resulting in a charge that is base-dependent.

A phosphodiester is a strong acid whose pK is around 1 [3]. Adenine (A) and cytosine (C) bases can be found as neutral or positive forms, whereas thymine (T) is neutral or negative, and guanine (G) can exist in all three states [3]. The ionization of the bases and their corresponding dissociation constants may be described by the equilibria reported in Table 1.

Table 1
Ionization equilibria for the four different bases found in DNA [3]

Base	Equilibrium	Equilibrium constant
Adenine	$AH^+ \leftrightarrow A + H^+$	$K_A = 10^{-3.5}$
Cytosine	$CH^+ \leftrightarrow C + H^+$	$K_C = 10^{-4.2}$
Thymine	$TH \leftrightarrow T^- + H^+$	$K_T = 10^{-9.2}$
Guanine	$GH_2^+ \leftrightarrow GH + H^+$	$K_{G1} = 10^{-2.1}$
	$GH \leftrightarrow G^- + H^+$	$K_{G2} = 10^{-9.2}$

2.2. Ionization of glass and of amine groups of silanized glass

For the description of charge and potential at the oxide/electrolyte interface, different models can be used. A common feature of these models is the notion of charging from dissociation of surface groups and a formulation of mass action laws applied to the solid/liquid interface. In the context of electrokinetic potential measurements, the triple layer model, which comprises a well-confined electric double layer close to the surface and a diffuse layer described by the Gouy–Chapman theory, is considered to be the best tool for a quantitative description [4]. Close to the solid surface, a well-confined electric double layer, also known as the Helmholtz layer, is formed. This is followed by a diffuse layer of counter charges between the outer Helmholtz plane and the neutral bulk of the solution. While the Helmholtz planes are strongly adsorbed, the diffuse layer is considered more mobile.

Several studies show that a simple thermodynamic analysis of the adsorption or desorption of protons allows us to relate the change in the solid/electrolyte interface free energy to the charge density at the oxide surface [5,6]. The well-confined electric double layer close to the solid surface is apparently sufficient to interpret contact angle titration methods, where the point of zero charge of the solid is the unknown parameter. However, simple Helmholtz double-layer description appears to be particularly well adapted to the case of a silanized glass substrate, as shown in this study.

As a first approximation, we assume that a glass surface behaves similarly to an amorphous silica surface. The estimated maximum coverage of silanols on a silica surface is about 4.5 SiOH groups/nm^2 [7]. When silica is submitted to a heat treatment, the number of free surface silanol groups is reduced [7].

SiOH groups are positively or negatively charged, according to the pH. The point of zero charge, pzc, defines the pH at which the surface density of positive charges is equal to the surface density of negative charges [8]. The pzc of silica is around pH 2 to 3 [8,9]. The pK_a of silanol groups is in the range from 5 to 7 [10,11].

We may calculate the ionic species present on a bare glass surface as a function of pH from the average pK_a value of 6 and from the pzc value. These lead to the following equations for the relative density of negative and positive species:

$$\mathrm{SiOH} \leftrightarrow \mathrm{SiO^-} + \mathrm{H^+}, \qquad K_1 = \frac{|\mathrm{SiO^-}||\mathrm{H^+}|}{|\mathrm{SiOH}|} = 10^{-6}, \quad (1)$$

$$\mathrm{SiOH_2^+} \leftrightarrow \mathrm{SiO^-} + 2\mathrm{H^+},$$

$$K = \frac{|\mathrm{SiO^-}||\mathrm{H^+}|^2}{|\mathrm{SiOH_2^+}|} = 10^{-2\mathrm{pzc}}, \quad (2)$$

$$\mathrm{SiOH_2^+} \leftrightarrow \mathrm{SiOH} + \mathrm{H^+}, \qquad K_2 = \frac{|\mathrm{SiOH}||\mathrm{H^+}|}{|\mathrm{SiOH_2^+}|} = \frac{K}{K_1}. \quad (3)$$

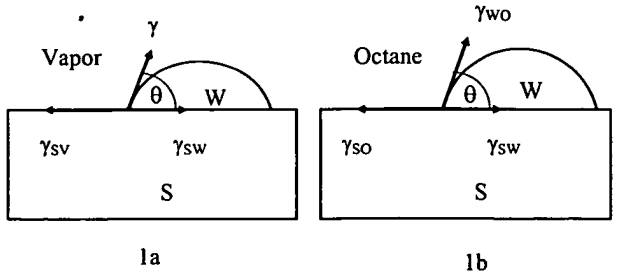

Fig. 1. Young's model for a sessile water drop in the presence of water vapor (a) or octane (b).

The equilibrium between protonated amine groups ($R–NH_3^+$) and amine groups ($R–NH_2$) is given by

$$R–NH_3^+ \leftrightarrow R–NH_2 + H^+, \qquad K_a = \frac{|R–NH_2||H^+|}{|R–NH_3^+|}. \tag{4}$$

The value of the equilibrium constant K_a may be estimated as being on the order of the quoted K_a value for N-propylamine, i.e., 5.78×10^{-11} ($pK_a = 10.2$). It is known that amine groups of APTS molecules are positively charged up to pH 10, which is equal to the pK_a value [12].

It has been demonstrated that only 30 to 50% of silanol groups may be grafted during silanization reaction [13]. Therefore, the chemical groups present on a silica (glass) surface silanized with APTS are likely to be amine groups and SiOH groups. We will see that our results are in agreement with this description.

2.3. Wettability at various pH

The equilibrium contact angle, θ, of a water sessile drop satisfies Young's equation,

$$\gamma_{SV} = \gamma_{SW} + \gamma\cos\theta, \tag{5}$$

where γ_{SV}, γ_{SW}, and γ are the solid surface free energy of the solid in presence of the water vapor, the interface free energy between water and the solid, and the water surface tension, respectively (Fig. 1a).

When an ionizable solid surface is in contact with an aqueous solution, it acquires a certain charge because of adsorption or desorption of a certain number of protons [5,6]. The water surface tension can be considered as constant when hydrochloric acid or sodium hydroxide is added to modify the pH [14]. Therefore, when the pH of water is changed, the wetting angle, θ, changes accordingly:

$$-d\gamma_{SW} = \gamma\, d(\cos\theta). \tag{6}$$

The surface charge, σ, may be considered as resulting simply from the adsorption of protons (H^+) if the solid surface is positively charged and from the adsorption of OH^- if the solid surface is negatively charged [5]. Using the Gibbs adsorption equation it can be deduced that

$$d\gamma_{SW} = -\Gamma_{H^+} RT\, d\ln|H^+| = 2.303\Gamma_{H^+} RT\, d(\text{pH}), \tag{7}$$

where Γ_{H^+} is the surface excess concentration of protons. The surface charge density, σ, of the water/solid interface is related to Γ_{H^+} by

$$\sigma = \Gamma_{H^+} F, \tag{8}$$

where F is the Faraday constant. Hence, Eqs. (6)–(8) yield

$$\frac{d(\cos\theta)}{d(\text{pH})} = -\frac{2.303RT\sigma}{F\gamma}, \tag{9}$$

indicating that the change in θ is controlled by σ. Note that the same Eq. (9) but with the right-hand side of opposite sign, is obtained for a negatively charged surface, due to the adsorption of OH^- groups. At the point of zero charge, pzc, $\sigma = 0$:

$$\frac{d(\cos\theta)}{d(\text{pH})} = 0. \tag{10}$$

Therefore, in the absence of specifically adsorbed ions, a maximum in θ or a minimum in $\cos\theta$ will occur at pzc as the pH of the water drop is scanned [5,6,14].

2.4. Wettability under octane

To measure the wettability as a function of pH for bare glass, the substrate was immersed in liquid octane (Fig. 1b). Under these conditions, the contact angle of water in (nonpolar) octane is only a function of specific, nondispersive, interactions between water and the substrate. This arises from water and octane having the same dispersive or London contribution to their surface tension (21.6 mN m^{-1} for water, 21.3 mN m^{-1} for octane [15,16]).

Another advantage of measuring wettability of water in an octane medium is that finite values of the contact angle can be obtained with a high-surface-energy substrate. For example, clean glass measured in air does not give a finite measurable contact angle with water.

As demonstrated in Section 2.3, a maximum in θ or a minimum in $\cos\theta$ is expected at pzc when the pH of the water drop is changed [6,14].

The nondispersive interaction between water and the substrate, I_{SW}^{nd}, may be deduced from the Young and Dupré equations,

$$\gamma_{SO} = \gamma_{SW} + \gamma_{WO}\cos\theta, \tag{11}$$

$$\gamma_{Si} = \gamma_S + \gamma_i - I_{Si}^{d} - I_{Si}^{nd}, \tag{12}$$

where subscript S represents the solid, and subscript i the liquid phase (water, W or octane, O), and θ is the water contact angle under octane. I_{Si}^{d} is the solid/liquid dispersive interaction (the sum $I_{Si}^{d} + I_{SW}^{nd}$ in Eq. (12) corresponds to the solid/liquid work of adhesion as expressed in the Dupré equation). Since $I_{SW}^{d} \approx I_{SO}^{d}$ and $I_{SO}^{nd} = 0$, I_{SW}^{nd} satisfies

$$I_{SW}^{nd} \approx \gamma_W - \gamma_O + \gamma_{WO}\cos\theta, \tag{13}$$

where γ_W (72.8 mN m^{-1}), γ_O (21.3 mN m^{-1}), and γ_{WO} (51 mN m^{-1}) are respectively the water and octane surface

tensions and the water/octane interface tension. The last is not affected by the water pH [14].

From the nondispersive (hydrogen bond) energy of interactions, at pzc, expressed per unit of interface area, I_{SW}^{nd}, and from the molar energy of hydrogen bonds, E, on the order of 24 kJ/mol [17], we can estimate the number, n, of hydrogen bonds per unit interface area ($n \approx (I_{SW}^{nd}/E) \times 6.02 \times 10^{23}$), which corresponds to the surface density of hydroxyl groups on the solid surface.

3. Experimental methods

3.1. Sample preparation

The glass slides have a thickness of 1.1 mm and the format of microscope slides (1 × 3″). The bare glass slides were cleaned by pyrolysis in an oven to burn organic contamination. The pyrolyzed samples were held in a glass rack and placed in a glass container covered with aluminum foil and closed with a glass lid. Following removal from the oven, the glass samples remained clean for as long as 1 week, provided the lid was not opened. Following opening of the container, the clean glass samples were used within 5 min. The low level of organic contamination was checked by controlling that water spread fully on glass slides. The glass substrate was treated with APTS molecules by using a proprietary process leading to a uniform coating with thickness on the order of one monolayer. After coating, the glass slides were placed in sealed packaging filled with nitrogen to prevent carbonatation of amine functions from carbon dioxide in air. Colloidal gold staining (Colloidal Gold Total Protein Stain, Bio-Rad Laboratories) verified uniformity of the surface charge on the coating.

3.2. Determination of the pzc of substrates by wettability measurements

The surface charge of bare glass and APTS-treated glass has been probed by measuring the water contact angle as a function of pH.

Contact angle measurements were made using a Ramé–Hart contact angle goniometer. The average water contact angle as a function of pH was obtained from measurements on five different glass slides of both APTS-coated and uncoated glass. Two measurements were made on one sessile drop for each pH value and on each slide (10 contact angle measurements per pH value). The water drops had a volume of 2 µl. Water was purified by ionic exchange (Elgastat, UHP), leading to a resistivity of 18 MΩ cm, followed by organic removal.

To measure the wettability as a function of pH for bare glass, the substrate was first immersed in octane (octane > 99%, Aldrich). Water wetting measurements were made also on APTS-coated glass in air as a function of pH. Adding HCl or NaOH to water varied the pH of water drops. The pH values were controlled with a pH meter (Basic 20, Crison).

3.3. Measurement of DNA retention

DNA retention was studied on APTS glass slides with double-stranded DNA obtained from polymerase chain reaction (PCR). The DNA used is the Bacillus subtilis 2 (BS2) gene of 1600 base pairs, 10% of molecules being labeled with Cy3 dye to measure the retention by fluorescence.

The DNA molecules, dissolved in a water/ethylene glycol mixture (1 : 4 by volume), were printed on APTS glass slides with a pin spotter in the form of small spots (Cartesian Technologies, dot diameter ≈ 250 µm). The pH of the water part in the solvent mixture was varied from 1 to 12.

The amount of DNA bound to the substrate was evaluated from the fluorescent intensities of spots measured with a scanner (Genepix Scanner (Cy3-wavelength, $\lambda = 532$ nm)). Fluorescent intensities were measured before and after the printed slides were washed with a prehybridization solution (50 ml of water, 25 ml of formamide, 25 ml of SSC 20X, and 0.5 ml of SDS 10X). The printed slides were dried for 4 h at 80 °C, before and after rinsing. The DNA retention is measured as the ratio of the fluorescent intensities before and after washing.

4. Results and discussion

4.1. Charge of single-stranded DNA in solution

Considering that in each nucleotide of DNA, the phosphate group may lead to one negative charge and the bases lead to positive, negative, or neutral charges, a simple calculation of the net average charge per nucleotide as a function of pH is possible. Assuming a probability of presence of 25% for each base per nucleotide, the average net charge per nucleotide, C/N, may be estimated as

$$\begin{aligned}\mathrm{C/N} \approx {} & -\frac{1}{1+|\mathrm{H^+}|/K_{\mathrm{ph}}} + 0.25\frac{1}{1+K_{\mathrm{A}}/|\mathrm{H^+}|} \\ & + 0.25\frac{1}{1+K_{\mathrm{C}}/|\mathrm{H^+}|} - 0.25\frac{1}{1+|\mathrm{H^+}|/K_{\mathrm{T}}} \\ & + 0.25\frac{|\mathrm{H^+}|^2 - K_{\mathrm{G1}}K_{\mathrm{G2}}}{K_{\mathrm{G1}}K_{\mathrm{G2}} - K_{\mathrm{G1}}|\mathrm{H^+}| + |\mathrm{H^+}|^2}, \end{aligned} \tag{14}$$

where K_{ph} ($= 10^{-1}$) corresponds to the ionization of phosphodiester groups and K_{A}, K_{C}, K_{T}, K_{G1}, and K_{G2}, are the dissociation constants given in Table 1. The resulting charge as a function of pH is plotted in Fig. 2. Equation (14) predicts that a single strand of DNA becomes negatively charged between pH 1 and pH 2. The solid line of Fig. 2 represents the contribution of phosphate groups, which are negatively charged from pH 0. This line may be considered as representing the charge of a double-stranded DNA polymer.

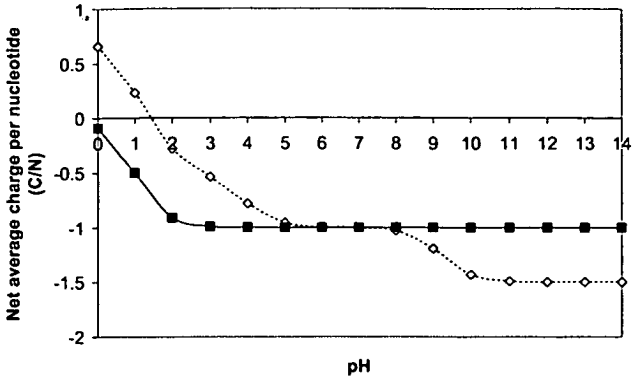

Fig. 2. Calculated net average charge per nucleotide of a double- or single-stranded DNA molecule in solution. The solid line represents the charge of a double-stranded DNA (phosphate groups). The dotted line represents the charge of a single-stranded DNA.

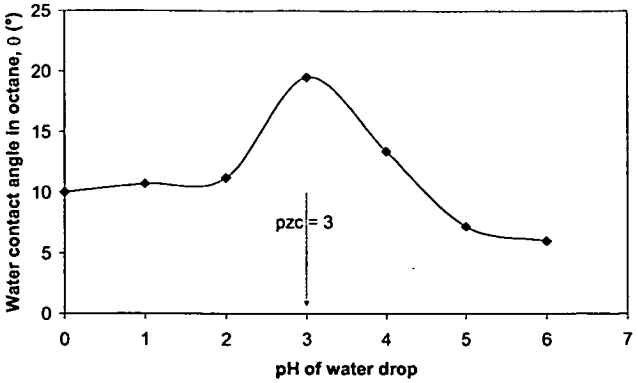

Fig. 3. Determination of the point of zero charge, pzc, of bare glass.

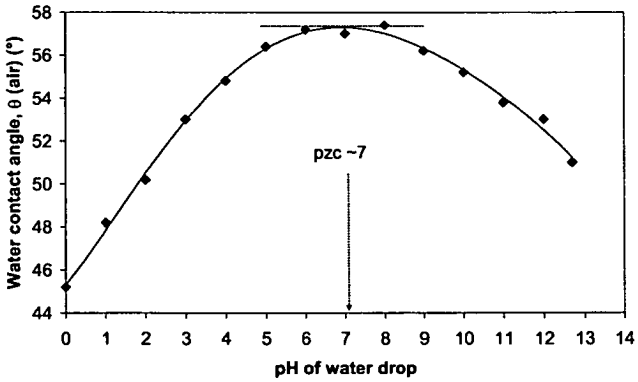

Fig. 4. Determination of the point of zero charge, pzc, of APTS slides by wettability measurements. The maximum of the wetting angle corresponds to the pzc (at pH ≈ 7) of the surface.

In fact, the presence of phosphates increases all the dissociation constants of the bases by a few tenths of a pH unit. This shift is due to the electrostatic attraction between the phosphate groups and any positively charged species, combined with the repulsion between the phosphate groups and any negatively charged species [3].

Above pH 2, a single strand of DNA may be considered as negatively charged. This is probably also true for double-stranded DNA, due to the dominant contribution of phosphate groups. The molecules may be seen as acidic polyelectrolytes.

4.2. Bare glass pzc

Figure 3 shows the variation of the water contact angle in octane as a function of the pH for bare glass. We observe that the wetting angle is clearly higher at pH 3, indicating a point of zero charge, pzc, at approximately pH 3. This pzc for a bare glass surface is close to that for a silica surface [8–12,15]. Therefore, we deduce that the equilibrium constant of silanol protonation (Eq. (3)) is on the order of 1.

We may deduce the ionic species present on bare glass as a function of pH from the equilibrium constants of dissociation or protonation of SiOH groups. It may be deduced that 50% of silanol groups are positively charged at pH 0. At pH 6, 50% of the silanol groups are negatively charged. Between these two values, the ionization of the silanol groups is weak. The fraction of positively and negatively charged silanol groups is on the order of 0.1% at pH 3.

The water contact angle in octane may be also used to estimate the density of silanol groups at the glass surface. The number of charged SiOH groups is very limited at the pzc (0.2%) and the contact angle in octane is 19.5°. We may consider that nondispersive interaction between water and the noncharged glass surface, I_{sw}^{nd}, at pH 3 is primarily generated by hydrogen bonding. From the nondispersive (hydrogen bonds) energy of interactions expressed per unit of interface area, I_{sw}^{nd}, and from the molar energy of hydrogen bonds, E, of 24 kJ/mol, the number, n, of hydrogen bonds per unit interface area ($n \approx (I_{sw}^{nd}/E) \times 6.02 \times 10^{23}$) is obtained. This gives about 2.5 silanol groups per square nanometer on the glass surface. This value is comparable to the density of silanol groups measured on silica that has been heat-treated at 500 °C [7].

4.3. Pzc of silanized glass

The variation of the water contact angle in air as a function of the pH of the water drop is presented in Fig. 4. The maximum of the contact angle on APTS slides is obtained at pH ≈ 7, implying that the pzc of the APTS treated glass is around pH 7. This result is in perfect agreement with the study of Golub et al. [18] on silica gels modified with APTS.

We cannot consider that the APTS surface is just a cover of amine functions, since amine functions are positively charged up to pH ≈ 11. Neutrality of the APTS surface at pH 7 implies the presence of negatively charged species. The SiO^- species from SiOH are a probable candidate.

We model the surface charge of an APTS slide by considering that it is composed of amine and silanol groups. The number of ionizable surface sites, c, is considered constant. We define α as the fraction of amine groups and $(1-\alpha)$ as the fraction of silanol groups. At the pH corresponding to the

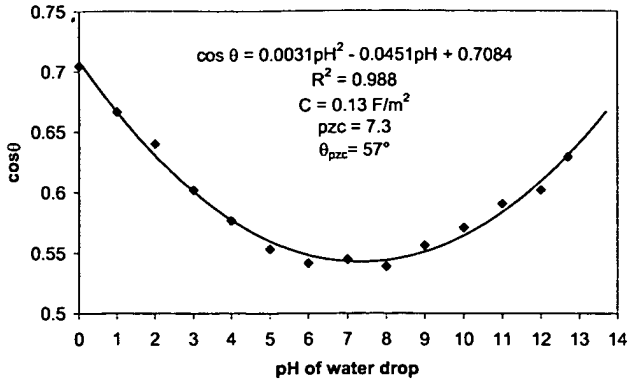

Fig. 5. Parabolic variation of the cosine of the water contact angle on the APTS-treated glass as a function of the pH of the water drop. The solid line represents a parabolic fit of experimental data.

pzc, the densities of positive and negative charges are equal:

$$|\mathrm{SiO^-}| = |\mathrm{SiOH_2^+}| + |\mathrm{R\text{–}NH_3^+}|. \tag{15}$$

At pH 7, the fraction of positively charged silanol groups is negligible. Therefore, Eq. (15), when combined with Eqs. (1) and (4), becomes

$$|\mathrm{SiO^-}| = \frac{(1-\alpha)c}{1+10^{\mathrm{p}K_1-7}} = |\mathrm{R\text{–}NH_3^+}| = \frac{\alpha c}{1+10^{7-\mathrm{p}K_a}}. \tag{16}$$

Solving this equation yields $\alpha = 0.48$. This is in good agreement with reported studies of APTS on silica [13]. This value of α implies that the surface densities of amine and silanol groups are about equivalent on APTS-treated glass. The amine density on the APTS substrate is therefore of the order of $0.48 \times 2.5 = 1.2$ groups per nm^2.

The graph in Fig. 5 gives the variation of the cosine of the water contact angle as a function of pH. It appears qualitatively that the curve has a parabolic shape and is symmetrical about the pzc. This curve is compatible with the constant capacitance model formed by an electrical double layer.

If the double layer forms a capacitor of capacitance C (per unit area), the surface charge satisfies

$$\sigma = CV, \tag{17}$$

where V is the surface potential. The energy of the capacitor, E, contributes to the water/solid interface free energy, so that the variation of the interface free energy may be also written as

$$dE = -d\gamma_{\mathrm{SW}} = d\left(\frac{1}{2}CV^2\right) = \sigma\, dV. \tag{18}$$

This is equivalent to the Lippman equation in electrowetting. Considering that $V = 0$ at pzc, combining Eqs. (6), (7), and (18) leads to the variation of the cosine of the wetting angle with the pH of water:

$$\cos\theta = \cos\theta_{\mathrm{pzc}} + \frac{CV^2}{2\gamma}$$

$$= \cos\theta_{\mathrm{pzc}} + \frac{C}{2\gamma}\left(2.303\frac{RT}{F}\right)^2(\mathrm{pzc} - \mathrm{pH})^2. \tag{19}$$

Equation (19) justifies the parabolic variation with pH of the cosine of the wetting angle of water on the APTS-treated glass. The solid line of Fig. 5 represents the parabolic fit of the experimental points. R^2, the correlation coefficient, is very close to 1 (0.988). From the constant term of the parabolic fit, it is possible to determine precisely the pzc of the solid surface as pH 7.3 and the value of the wetting angle at pzc of 57°. The similarity with electrowetting becomes evident if θ_{pzc} is considered as equivalent to $\theta_{V=0}$ in electrowetting.

Hence, the surface charge of the APTS-treated glass leads to the formation of an electrical double-layer, comparable to the Helmholtz double-layer description, when placed in contact with water. The simple model of a constant capacitor for this double layer is in good agreement with the experimental results. The value of the constant capacitance is found to be equal to 0.13 F/m^2. Considering a value of 80 for the water dielectric constant leads to a distance d between the inner and outer plane of the double layer of 5.5 nm ($d = \varepsilon_0\varepsilon_r/C$). The pzc at pH 7.3, instead of pH 7, does not significantly alter the fraction of amine functions of the total ionizable surface sites (0.49 versus 0.48).

4.4. DNA retention as a function of the proton concentration

The DNA retention, expressed as a fraction of the amount of DNA retained by the substrate after washing, appears to be strongly dependent on the proton concentration of the printing solution, as shown in Fig. 6. An increased DNA retention is observed at the highest proton concentrations. The four curves correspond to four different APTS slides with four printed DNA dots per proton concentration value.

We can deduce that a positively charged substrate is favorable to having a high retention of printed DNA. This effect occurs despite the fact that the dry DNA spots are then washed by the same buffer solution.

At high proton concentrations, between 10^{-1} and 10^{-2} mol/l, the phosphate groups of DNA are negatively charged

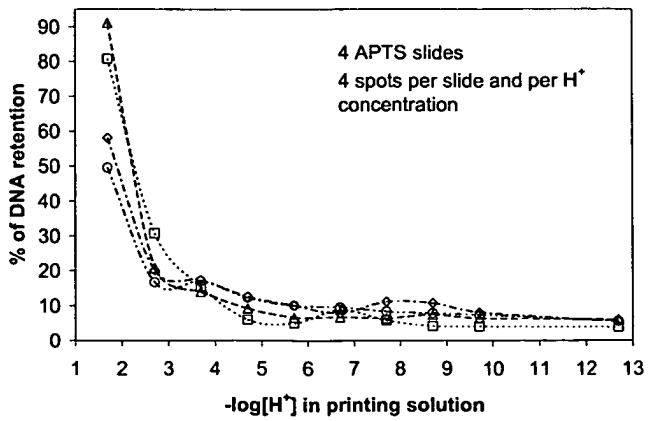

Fig. 6. DNA retention as a function of the proton concentration in the DNA printing ink.

(Fig. 2). The silanol groups become positively charged, increasing the net positive charge of the substrate. Thus, it is likely that the increase of DNA retention at very high proton concentrations is due to ionic interactions between negatively charged phosphate groups of DNA and the positively charged amine and silanol groups of the substrate.

Therefore, the DNA printing conditions, and in particular the proton concentration, have a strong impact on DNA retention, even after washing.

5. Conclusion

Wetting experiments performed at various pH reveal that a glass surface treated with APTS is positively charged below pH 7 and negatively charged above pH 7, although the amine groups alone are positively charged up to pH ≈ 11.

A simple model allows an estimate of the net surface charge of silica or glass treated with APTS. The APTS substrate is positively charged below pH 7 and negatively charged above pH 7, due to the contribution of amine and free silanol groups. The model indicates that the surface is composed of about 50% amine groups and 50% silanol functions. This result is in good agreement with the conclusions of former studies of the adsorption of APTS on silica [13,18]. The origin of these free silanol groups may be the glass surface or hydrolyzed alkoxy groups of the APTS silane.

A good correlation between the DNA retention data and the variation of the surface charge of APTS silanized glass has been obtained. The retention of DNA is improved when DNA spots are printed in acidic conditions, probably because phosphate groups of DNA are negatively charged from pH 0 and both the amine functions of APTS and the silanol groups are positively charged at this pH.

This study may be considered as an attempt to describe the ionic interactions between DNA molecules and functionalized substrates and their consequences on DNA retention. The subject is quite complex. It is evident that DNA may interact with the surface via other interactions, as hydrophobic interactions. However, our results show evidence of the important role played by ionic interactions in the retention of DNA molecules on aminated substrates, such as APTS-treated slides.

We have demonstrated that the electrical double layer formed at the interface between water and the APTS-treated glass is perfectly described by a model of constant capacitance of the double layer. The electrical behavior of the APTS-treated glass in contact with water is remarkable and it may be used to better control the retention of DNA and the properties of the functionalized substrate.

References

[1] V. Chan, S.E. McKenzie, S. Surrey, P. Fortina, D.J. Graves, J. Colloid Interface Sci. 203 (1998) 197.
[2] V. Balladur, A. Theretz, B. Mandrand, J. Colloid Interface Sci. 194 (1997) 408.
[3] C.R. Cantor, P.R. Schimmel, Biophysical Chemistry I. The Conformation of Biological Macromolecules, Freeman, New York, 1980.
[4] S. Koch, P. Woias, L.K. Meixner, S. Drost, H. Wolf, Biosensors Bioelectron. 14 (1999) 417.
[5] L.-K. Chau, M.D. Porter, J. Colloid Interface Sci. 145 (1991) 283.
[6] E. McCafferty, J.P. Wightman, J. Colloid Interface Sci. 194 (1997) 344.
[7] L.T. Zhuravlev, Colloids Surf. A Physicochem. Eng. Asp. 74 (1993) 71.
[8] G.A. Parks, Chem. Rev. 65 (1965) 177.
[9] A. Carré, F. Roger, C. Varinot, J. Colloid Interface Sci. 154 (1992) 174.
[10] M.L. Hair, W. Hertl, J. Phys. Chem. 74 (1970) 92.
[11] R.A. Van Wagenen, J.D. Andrade, J.B. Hibbs, J. Electrochem. Soc. 123 (1976) 1438.
[12] M. Bezanilla, S. Manne, D.E. Laney, Y. Lyubchenko, H.G. Hansma, Langmuir 11 (1995) 655.
[13] E.F. Vansant, P. Van der Voort, K.C. Vranken, Characterization and Chemical Modification of the Silica, in: Studies in Surface Science and Catalysis, Vol. 93, Elsevier, Amsterdam, 1995.
[14] C. Vittoz, M. Mantel, J.C. Joud, J. Adhesion 67 (1998) 347.
[15] W. Birch, S. Carré, A. Carré, in: Proceedings EURADH '2000, 5th European Adhesion Conference, 2000, p. 242.
[16] W. Birch, S. Mechken, A. Carré, in: K.L. Mittal (Ed.), Proceedings of the International Symposium on Surface Contamination and Cleaning, MST Conferences, Newark, NJ, 2001, to be published.
[17] J.N. Murrell, A.D. Jenkins, Properties of Liquids and Solutions, Wiley, New York, 1994.
[18] A.A. Golub, A.I. Zubenko, B.V. Zhmud, J. Colloid Interface Sci. 179 (1996) 482.

Journal of General Microbiology (1988), **134**, 1299–1305. *Printed in Great Britain*

Adherence of Streptococci to Surface-modified Glass

By N. SATOU,[1]* J. SATOU,[1] H. SHINTANI[1] AND K. OKUDA[2]

[1]*Department of Operative Dentistry and* [2]*Department of Biochemistry, Hiroshima University, School of Dentistry, Hiroshima 734, Japan*

(*Received 8 June 1987; revised 16 December 1987*)

Four types of surface-modified glass were prepared. Aminopropyl glass was prepared by alkylsilylation of glass slides with γ-aminopropyltriethoxysilane. This glass carries primary amino groups which may be protonated at pH 7·2. Owing to the presence of both positively charged ions and hydrophobic ethoxyl groups, the glass is considered to be amphipathic. Three other types of surface-modified glass slides were prepared from aminopropyl glass by forming Schiff's bases with three aldehydes: glucose, glyoxylic acid and hexanal. The aldehyde-treated slides were subsequently reduced using sodium borohydride. Thus, the surface of the glass was rendered hydrophilic, ampholytic or hydrophobic, respectively. The adherence of two *Streptococcus sanguis* strains and two *Streptococcus mutans* strains to the surface-modified glass slides was studied. Different strains showed differences in adherence to these slides depending on their physico-chemical surface properties. For *S. sanguis* ATCC 10556, hydrophobic bonds seemed to be most important, while in *S. mutans* OMZ 176, ionic interactions made the highest contribution to adhesion. Hydrogen bonds seemed to contribute least to adherence.

INTRODUCTION

Bacterial adherence to dental materials has been extensively studied (Rutter & Vincent, 1980; Gibbons & Van Houte, 1980). Some authors have attributed adherence to electrostatic forces (O'Brien *et al.*, 1978; Onose *et al.*, 1980; Larsson & Glantz, 1981; Reynold & Wong, 1983), while others have attributed it to hydrophobic interactions (Nesbitt *et al.*, 1982; Busscher *et al.*, 1984; Weerkamp *et al.*, 1985; Van Pelt *et al.*, 1985). A thermodynamic model was developed to establish a correlation between surface free energy and bacterial adherence on hydrophobic surfaces, assuming a constant contribution of electrostatic forces (Gerson, 1980). However, studies of the adherence of oral bacteria to dental prosthetics or restoratives have shown that the electrostatic contribution may vary from species to species. Furthermore, some authors have used gold plate as an electrode to which bacteria were attached (Onose *et al.*, 1980), while others used dental prosthetics as substrates (Minagi *et al.*, 1985). The use of such a variety of substrates has prevented the direct comparison of the relative contributions of individual physico-chemical forces to the adherence of oral bacteria to prosthetics or restoratives.

To study the mechanism of bacterial adherence to solid surfaces, the experimental system must be simplified so that the relative contribution of the various chemical and physical forces can be assessed. In this work, we prepared glass slides having various organic functional groups on the surface, such as amino, carboxyl, hydroxyl or hydrophobic groups attached through the covalent bond between silicon and carbon. Measuring the adherence of oral bacteria to these slides allowed a comparison of the relative contributions to adherence made by various physico-chemical forces.

Abbreviations: AmP-glass, aminopropyl glass; GA-, GL- and HA-glass, AmP-glass modified by conjugation with glyoxylic acid, D-glucose and hexanal, respectively (see text).

0001-4223

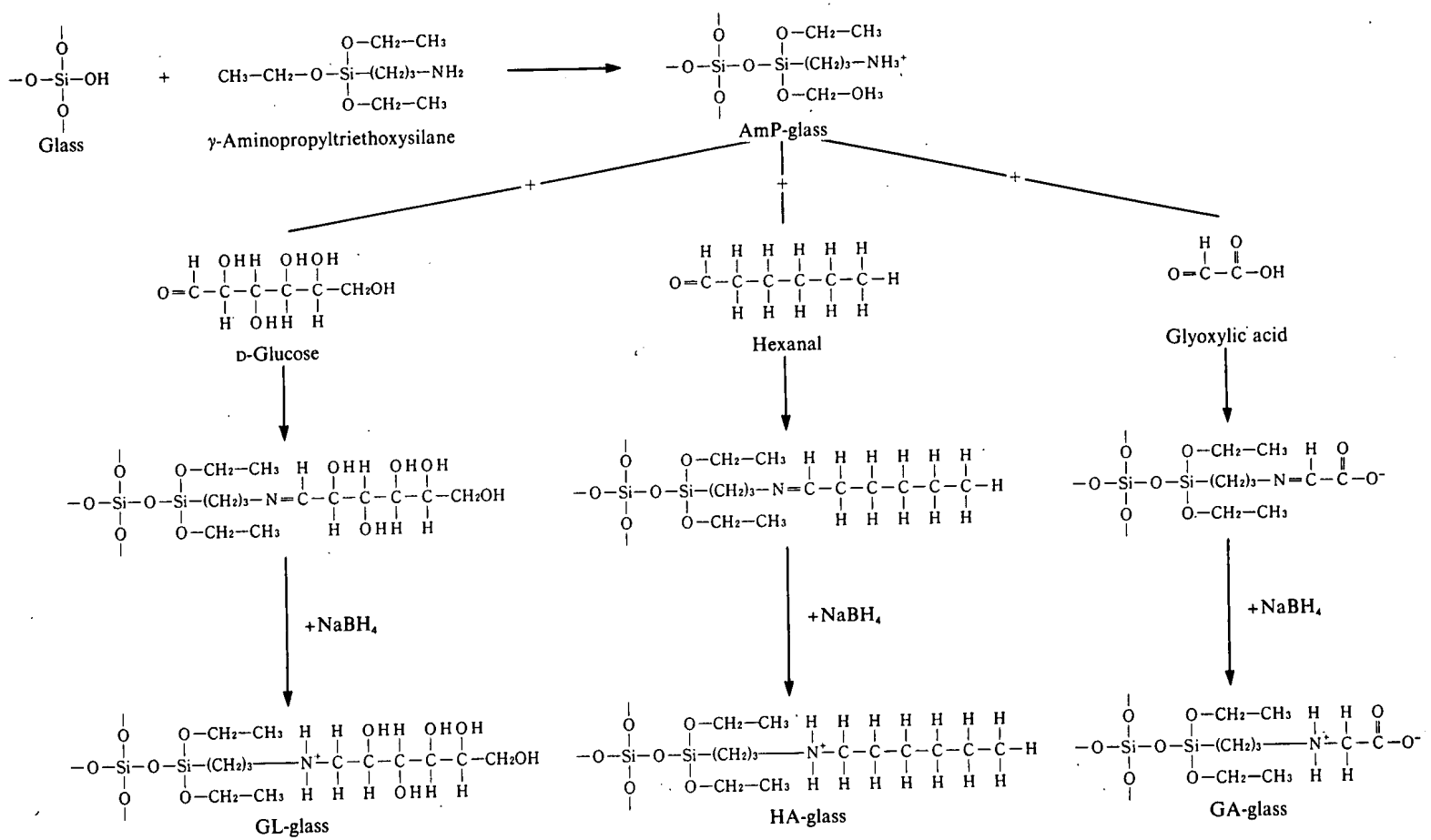

Fig. 1. Scheme for preparation of surface-modified glass slides.

Table 1. *Contact angles of distilled water on surface-modified glass slides*

The values are means of 36 determinations ± SD.

Modified glass	Contact angle
GL-glass	48·8 ± 6·0
HA-glass	75·1 ± 1·6
AmP-glass	69·5 ± 0·6
GA-glass	46·6 ± 3·5

METHODS

Preparation of aminopropyl (AmP) glass. Glass slides (Matunami Garasu Co., Kyoto, Japan) were immersed in potassium dichromate/sulphuric acid for 2 h, rinsed with running water, washed twice with distilled water and dried. The slides were then immersed in 10 M-NaOH for 24 h. After washing as described above, they were dried at 500 °C for 5 h. The slides were then treated with 4% (v/v) γ-aminopropyltriethoxysilane in acetone at 45 °C for 24 h according to the method described by Robinson *et al.* (1971) and Baum *et al.* (1972). After completion of the reaction, the slides were washed with acetone to remove excess reagent, air-dried and stored in a desiccator (these slides are referred to as AmP-glass). Since the primary amino groups may associate with protons at pH 7·2, the surface of AmP-glass may have a positive charge, and therefore be partially hydrophilic at this pH. However, since the rest of the γ-aminopropyldiethoxysilyl groups may be hydrophobic, AmP-glass is considered to be amphipathic. To estimate the amount of aminopropyl groups attached to AmP-glass slides, they were treated with 2,4,6-trinitrobenzenesulphonic acid (Cuatrecasas, 1970). The AmP-glass produced an orange colour product. The trinitrophenylated AmP-glass was cut into smaller pieces, which were put into a cuvette (light path 1 cm). The cuvette was filled with distilled water to make a final volume of 3 ml. The absorbance at 345 or 420 nm was determined against distilled water. The total amount of chromophore was calculated from the molar absorbancy index of trinitrophenylated glycine ($6{\cdot}51 \times 10^3$ cm^{-1} M^{-1} at 420 nm and $14{\cdot}1 \times 10^3$ cm^{-1} M^{-1} at 345 nm: Mokrasch, 1967). By dividing the total amount by the surface area of AmP-glass, a value of $1{\cdot}49 \times 10^{-3}$ μmol cm^{-2} for the coverage of amino groups per cm^2 on AmP-glass was obtained (it was assumed that aminopropyl groups on AmP-glass were in a monomolecular layer). Additional corroboration for the alteration of the glass surface was obtained by comparing the contact angle of water on the modified glass (69·5°; see Table 1) with that on untreated glass (25·2°).

Conjugation of glyoxylic acid, hexanal and D-*glucose to AmP-glass.* AmP-glass slides were treated with 5% (w/v) glyoxylic acid in distilled water, 5% (v/v) hexanal in tetrahydrofuran, or 5% (w/v) D-glucose in distilled water at room temperature for 2 h. After completion of the reactions, excess reagents were removed by washing with a large amount of distilled water (or, in the case of hexanal treatment, with absolute tetrahydrofuran and water), followed by treatment with 0·4% sodium borohydride in distilled water for 24 h at room temperature to reduce the imino group to the more stable amino group. The surface-modified slides so produced were washed with distilled water and used for the adherence assay. The modified slides thus formed are referred as GA-glass, HA-glass, GL-glass, respectively (Fig. 1), and their surface properties were ampholytic (hydrophilic), hydrophobic and hydrophilic, respectively. The concentrations of the attached groups (glucose, hexanal and glyoxylic acid residues) were not determined, since no convenient method to estimate these residues was available. Instead, the contact angle of water on each surface-modified glass was measured so as to judge whether the conjugation reaction had been successful (Table 1). The contact angle on HA-glass was higher than that on any of the other surface-modified glasses tested, consistent with the hydrophilic group in AmP-glass ($-NH_3^+$) having been converted into a more hydrophobic hexylamino group. A similar conclusion was reached for GA-glass. The lower contact angle for GA-glass than that for AmP-glass was consistent with the hydrophilic nature of the added ligand (glyoxylic acid). From these results it appeared that the Schiff's base forming reaction was at least partially successful.

Organisms and culture conditions. Streptococcus sanguis ATCC 10556, *S. sanguis* ATCC 10557, *S. mutans* OMZ 176 and *S. mutans* Ingbritt were used. All strains were grown in trypticase soy broth (BBL Microbiology Systems) supplemented with 0·5% yeast extract. The cells were harvested during the exponential growth phase by centrifugation at 1000 *g* at 4 °C for 15 min, and washed twice with 0·05 M-Tris/HCl buffer (pH 7·2). The washed bacteria were suspended in the same buffer. The cell suspensions were subjected to low-intensity ultrasonic treatment in tubes held on crushed ice, to disperse bacterial chains and aggregates (Stinson *et al.*, 1982). Generally, 40 s of minimum-intensity ultrasound was sufficient to produce predominantly single cells and pairs (Stinson *et al.*, 1982). Cell suspensions were immediately used for the experiment to avoid reaggregation. The optical densities of the suspensions were measured in a 3 ml cuvette with a 1 cm light path, using a Hitachi model 100-50 spectrophotometer, and the suspensions were adjusted to give an OD_{550} of 0·3 ($3{\cdot}65 \times 10^8$ cells ml^{-1}).

Fig. 1. Scheme for preparation of surface-modified glass slides.

Adherence test. Six slides of each surface-modified glass (all prepared from AmP-glass made at one time) were placed in a beaker, making a circle round the central area occupied by a magnetic stirring rod. Bacterial suspensions were poured into the beaker and stirred gently for 2 h at 37 °C. Each slide was taken out, washed with 50 ml distilled water in a beaker, fixed with 2·5% (v/v) glutaraldehyde at 4 °C for 30 min, and stained with 1% (w/v) acridine orange. The numbers of adherent bacteria were obtained by counting bacteria directly under a fluorescence microscope (Olympus model BHS) according to the method described by Ørstavik *et al.* (1974). The numbers of bacteria in each of 20 separate fields were counted for every slide and divided by the area of the field. From the values thus obtained the average number of cells per mm^2 of glass-surface was calculated. The mean values of these averages, obtained from six replicate slides of each surface-modified glass, were calculated with their standard errors. All the numerical data obtained were subjected to Student's *t*-test at the 1% and 5% levels. Each strain was tested four times in this system.

Determination of contact angle. The contact angles of distilled water on the four types of surface-modified glass were measured by the horizontal projection technique with a contact-angle meter (model CA-A; Kyowa Co., Tokyo, Japan) at 20 °C and at six separate points on six slides of each type of modified glass.

To measure the contact angle of streptococci, a streptococcal layer was prepared on a membrane filter (pore size 0·45 μm; Millipore) from 10 ml of bacterial suspension with an OD_{550} of 1·0 (10^{10} cells per 10 ml). The filter carrying the streptococcal layer was air-dried for 120 min (Busscher *et al.*, 1984), and the contact angle of water was measured by the same method as that for surface-modified glass.

Determination of zeta potential. Bacterial suspensions were prepared as described above. Electrophoresis was performed at the so-called stationary level in a particle micro-electrophoresis apparatus, using a flat glass cell and platinum electrodes (Olsson *et al.*, 1976). The voltage used was 10 V. The electrokinetic potential (ζ) was calculated from the Helmholtz–Smoluchowski formula (Davies & Rideal, 1963): $\zeta = 4\pi\eta U/D$, where U is the electrophoretic mobility or velocity at unit potential gradient, η is the viscosity of the liquid, and D is the dielectric constant.

RESULTS

Effect of time on adherence of bacteria to AmP-glass. The adherence assay was carried out for varying time periods. The numbers of bacteria adhering to each kind of glass increased with time until they reached a saturation level which was specific for each type of glass (Fig. 2). After 2 h the numbers of bacteria adhering remained constant. In all the following experiments 2 h incubations were used.

Adherence of streptococcal strains to the various surface-modified glasses. Table 2 shows the results obtained by incubating the two *S. sanguis* strains and two *S. mutans* strains with the four different kinds of surface-modified glass. The numbers of bacteria adhering varied, depending on the type of surface-modified glass. *S. sanguis* ATCC 10556 adhered in highest numbers to HA-glass and in lowest numbers to GL-glass ($P < 0{\cdot}01$). There was no significant difference between its adherence to GA-glass and to GL-glass. With *S. sanguis* ATCC 10557 no such difference between HA-glass and GL-glass was observed, suggesting that different strains of a single species of bacteria may have different surface properties. In contrast, both *S. mutans* strains showed the highest adherence capacity to AmP-glass of the four types of surface-modified glass, suggesting an important contribution of electrostatic force in adherence of these strains. Although both *S. mutans* strains adhered to HA-glass, there was a significant difference ($P < 0{\cdot}01$) in their affinity for this glass, suggesting that the relative contribution of hydrophobic bonds might be different between these two strains.

Contact angles of the streptococcal strains. The contact angles of water droplets on dried bacterial surfaces for the four strains (means ± SD, $n = 10$) were: *S. sanguis* ATCC 10556, 37·2° ± 1·4°; *S. sanguis* ATCC 10557, 44·1° ± 2·8°; *S. mutans* OMZ 176, 32·0° ± 0·8°; *S. mutans* Ingbritt, 15·8° ± 2·2°.

ζ-potentials of the streptococcal strains. Although the ζ-potentials of many oral bacteria were studied by Olsson *et al.* (1976), they were determined using a different buffer and pH from those adopted in this experiment. To correlate the present adhesion and contact-angle results with the ζ-potentials of the bacteria, we thus determined the ζ-potentials under the conditions used for our experiments. The ζ-potentials of the four strains fell in a range between −21 and −32 mV (Table 3), values consistent with those measured by Olsson *et al.* (1976). The less negative ζ-

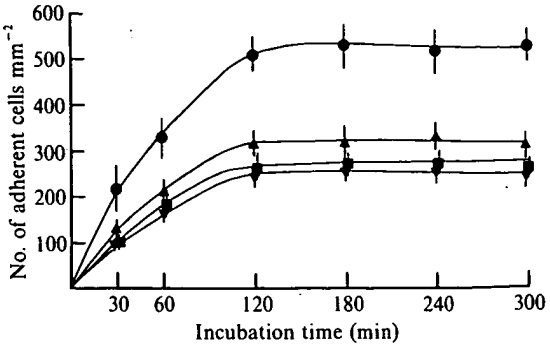

Fig. 2. Effect of time on adherence of *S. sanguis* ATCC 10556 to surface-modified glass (▲, AmP-glass; ■, GA-glass; ▼, GL-glass; ●, HA-glass). Vertical lines indicate standard deviations obtained from six replicate glass slides.

Table 2. *Adherence of oral streptococci to various surface-modified glass slides*

The values indicate the mean number (± SE) of bacteria adhered per 1 mm² of various surface-modified glass slides (the values were obtained from six replicate slides, for each of which the number of bacteria adhered in 20 separate views was counted and the mean number of bacteria adhered per mm² was calculated). Differences between the values indicated by the same letter are significant at the 5% level (*c, e, s, v, w*) or the 1% level (*a, b, d, f, g, h, k, t*).

Modified glass	*S. sanguis* ATCC 10556	*S. sanguis* ATCC 10557	*S. mutans* OMZ 176	*S. mutans* Ingbritt
GL-glass	132 ± 4^{a}	593 ± 20^{e}	353 ± 15^{fg}	256 ± 25^{tv}
HA-glass	485 ± 35^{ab}	613 ± 11^{d}	582 ± 44^{fhs}	367 ± 32^{v}
AmP-glass	210 ± 21^{ac}	571 ± 16	760 ± 42^{kgs}	418 ± 25^{tw}
GA-glass	150 ± 8^{bc}	521 ± 16de	404 ± 23^{kh}	295 ± 29^{w}

Table 3. *Electrophoretic mobility (towards the anode) and ζ-potential of oral streptococci in Tris/HCl buffer (0·05 M, pH 7·2) at 25 °C*

Strain	Electrophoretic mobility* (μm.cm s^{-1} V^{-1})	ζ-potential† (mV)
S. sanguis ATCC 10556	1·89 ± 0·29	−24·4
S. sanguis ATCC 10557	1·66 ± 0·18	−21·4
S. mutans OMZ 176	2·42 ± 0·20	−31·0
S. mutans Ingbritt	2·46 ± 0·13	−31·7

* The mean values and standard deviations of each strain were calculated from the mobilities of 10 cells in two directions measured on five occasions.

† Calculated from the mean electrophoretic mobilities according to the Helmholtz–Smoluchowski formula.

potentials of the two strains of *S. sanguis* as compared with the two *S. mutans* strains is consistent with the data on contact angles, where *S. sanguis* showed larger contact angles (more hydrophobic) than *S. mutans*.

DISCUSSION

When the aldehyde group of hexanal was used to make a Schiff's base link with the amino group of AmP-glass, the surface of the resultant HA-glass seemed to be rendered more hydrophobic due to the introduction of the hexyl group. The *S. sanguis* strains showed the highest affinity to HA-glass, suggesting that hydrophobic bonds play an important role in their

adherence, although the extent of the contribution may vary according to the nature of the bacterial cell surface. Thus, in *S. sanguis* ATCC 10556 hydrophobic bonds seemed to make the largest contribution, while in *S. mutans* Ingbritt they seemed to contribute to a lesser extent. This is consistent with the observation that *S. sanguis* ATCC 10556 showed a high contact angle (indicating a highly hydrophobic surface), whereas *S. mutans* Ingbritt showed much lower contact angle (indicating a less hydrophobic surface).

AmP-glass possesses positively charged groups ($-NH_3^+$) and GA-glass possesses both negatively charged ($-COO^-$) and positively charged ($-NH_2^+-$) groups. The fact that AmP-glass showed a higher adherence capacity for *S. mutans* than GA-glass is consistent with the strongly negative ζ-potentials found for *S. mutans*. The generally lower adherence to GA-glass than to AmP-glass could be due to the repulsive force between the negative charges on the GA-glass and the bacterial surface.

The fact that for the two strains of *S. mutans*, the numbers of bacteria adhering to AmP-glass were higher than those adhering to HA-, GA- and GL-glasses suggests that electrostatic interaction may be important in the adherence of these strains. Conversely, the lower adherence of *S. sanguis* ATCC 10556 to AmP-glass than to the other types of glass tested suggests that in this strain electrostatic interactions contribute to a lesser extent than hydrophobic interactions. From these results it may be concluded that in *S. sanguis* hydrophobic interactions play a more important role than electrostatic forces, while in *S. mutans* the reverse is true.

GL-glass showed the lowest adherence capacity for all the bacteria tested except *S. sanguis* ATCC 10557. Since the carbonyl group of glucose was used to make the Schiff's base link, the surface of the resultant GL-glass was rendered much more hydrophilic by the addition of penta-hydroxymethylene residues. The hydroxyl group possesses a strong capacity to form hydrogen bonds if there are acceptors or donors present. Water molecules may function as acceptors or donors, and a strong interaction between GL-glass and water molecules may have prevented the adherence of bacterial cells.

From these results, it may be concluded that neither electrostatic forces alone nor hydrophobic interactions alone determine the adherence of *S. sanguis* and *S. mutans* to modified glass.

Since the method presented here is a model, the results obtained may not be directly applicable to bacterial adherence in the oral cavity. However, studies with glass surfaces covalently modified with hydrophobic, hydrophilic, and/or charged groups provide a useful technique for estimating the relative strengths of physico-chemical forces working on non-specific bacterial adherence, and may be applied to the development of materials resistant to bacterial adherence.

REFERENCES

BAUM, G., WARD, F. B. & WEETTALL, H. H. (1972). Stability, inhibition and reactivation of acetylcholinesterase covalently coupled to glass. *Biochimica et biophysica acta* **268**, 411–414.

BUSSCHER, H. J., WEERKAMP, A. H., VAN DER MEI, H. C., VAN PELT, A. W. J., DE JONG, H. P. & ARENDS, J. (1984). Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. *Applied and Environmental Microbiology* **48**, 980–983.

CUATRECASAS, P. (1970). Protein purification by affinity chromatography. *Journal of Biological Chemistry* **245**, 3059–3065.

DAVIES, J. T. & RIDEAL, E. K. (editors) (1963). *Interfacial Phenomena*. New York: Academic Press.

GERSON, D. F. (1980). Cell surface energy, contact angles and phase partition – lymphocytic cell lines in biphasic aqueous mixtures. *Biochimica et biophysica acta* **602**, 269–280.

GIBBONS, R. J. & VAN HOUTE, J. (1980). Bacterial adherence and the formation of dental plaque. In *Bacterial Adherence, Receptors and Recognition*, series B, vol. 6, pp. 61–104. Edited by E. H. Beachey. New York & London: Chapman & Hall.

LARSSON, K. & GLANTZ, P. O. (1981). Microbial adhesion to surfaces with different surface charges. *Acta odontologica scandinavica* **39**, 79–82.

MINAGI, S., MIYAKE, Y., INAGAKI, K., TSURU, H. & SUGINAKA, H. (1985). Hydrophobic interaction in *Candida albicans* and *Candida tropicalis* adherence to various denture base resin materials. *Infection and Immunity* **47**, 11–14.

MOKRASCH, L. C. (1967). Use of 2,4,6-trinitrobenzenesulfonic acid for the coestimation of amines, amino acids, and proteins in mixtures. *Analytical Biochemistry* **18**, 64–71.

NESBITT, W. E., DOYLE, R. J. & TAYLOR, K. G. (1982). Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxyapatite. *Infection and Immunity* **38**, 637–644.

O'BRIEN, W. J., FAN, P. L., LOESCHE, W. J., WALKER, M. C. & APOSTOLIDS, A. (1978). Adsorption of

Streptococcus mutans on chemically treated hydroxyapatite. *Journal of Dental Research* **57**, 910–914.

OLSSON, J., GLANTZ, P. O. & KRASSE, B. (1976). Electrophoretic mobility of oral streptococci. *Archives of Oral Biology* **21**, 605–609.

ONOSE, H., MIYAZAKI, T. & NOMOTO, S. (1980). Effect of electrical potential on the adherence of *Streptococcus sanguis* to gold plate. *Journal of Dental Research* **59**, 1179.

ØRSTAVIK, D., KRAUSE, F. W. & HENSHAW, L. C. (1974). *In vitro* attachment of streptococci to the tooth surface. *Infection and Immunity* **9**, 794–800.

REYNOLD, E. C. & WONG, A. (1983). Effect of absorbed protein on hydroxyapatite zeta potential and *Streptococcus mutans* adherence. *Infection and Immunity* **39**, 1285–1290.

ROBINSON, P. J., DUNNILL, P. & LILLY, M. D. (1971). Porous glass as a solid support for immobilisation or affinity chromatography of enzymes. *Biochimica et biophysica acta* **242**, 659–661.

RUTTER, P. R. & VINCENT, B. (1980). The adhesion of microorganisms to surfaces: physico-chemical aspects. In *Microbial Adhesion to Surfaces*, pp. 79–92. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter & B. Vincent. Chichester: Ellis Horwood.

STINSON, M. W., LEVINE, M. J., CAVESE, J. M., PRAKOBPHOL, A., MURRAY, P. A., TABAK, L. A. & REDDY, M. S. (1982). Adherence of *Streptococcus sanguis* to salivary mucin bound to glass. *Journal of Dental Research* **61**, 1390–1393.

VAN PELT, A. W. J., WEERKAMP, A. H., UYEN, M. H. W. J. C., BUSSCHER, H. J., DE JONG, H. P. & ARENDS, J. (1985). Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies. *Applied and Environmental Microbiology* **49**, 1270–1275.

WEERKAMP, A. H., VAN DER MEI, H. C. & BUSSCHER, H. J. (1985). The surface free energy of oral streptococci after being coated with saliva and its relation to adhesion in the mouth. *Journal of Dental Research* **64**, 1204–1210.

Proteomic Studies Using Microarrays

Tanja Feilner[1,3], Jürgen Kreutzberger[1], Birgit Niemann[2], Armin Kramer[1], Alexandra Possling[1], Harald Seitz[1], Birgit Kersten[1,4,*]

[1]*Max Planck Institute for Molecular Genetics, Department Lehrach, Ihnestrasse 73, D-14195 Berlin, Germany;* [2]*Federal Institute for Risk Assessment, Thielallee 88-92, D-14195 Berlin, Germany;* [3]*Present address: Centre for Human Proteomics, Royal College of Surgeons in Ireland, Dublin 2, Ireland and* [4]*Present address: Max Delbruck Center for Molecular Medicine, Department Neuroproteomics, Robert-Rossle-Str. 10, 13092 Berlin, Germany.*

Abstract: Microarray technology plays an increasing role in proteomic research. We give an overview about recent developments in this technology focusing on molecular interaction studies using protein and antibody microarrays. We report about technical aspects in the development of protein microarrays and describe different surfaces and detection modes. Furthermore, we review the applications of protein microarrays in different molecular interaction studies including interactions of proteins with antibodies, proteins, DNA, small molecules and enzymes. Advantages and limitations of the microarray-based methods with other *in vitro* methods have been compared. We present the increasing applications of protein and antibody microarrays in basic research, diagnostics, drug discovery, and *in vitro*-risk assessment of nutrients.

Key Words: Protein microarray, antibody microarray, protein chip, molecular interaction, enzymatic assay, serum profiling, diagnostics, drug discovery, chemical genomics.

1. INTRODUCTION

As an immediate consequence of the large-scale genomic sequencing efforts, a strong interest has emerged in analyzing the function of the DNA-encoded information. In striking contrast to the estimated 30,000 – 50,000 genes (Lander *et al.*, 2001; Venter *et al.*, 2001), the complexity of the human proteome is expected to range from 100,000 to several million protein molecules (Cahill, 2001; Laurell and Marko-Varga, 2002). This is mainly due to post-transcriptional control of protein translations (McCarthy, 1998) and over 200 possible post-translational modifications of proteins (Meri and Baumann, 2001; Parekh and Rohlff, 1997). With respect to protein concentration, the situation is additionally complicated by the fact that the dynamic range can be as large as 10^6 (Pandey and Mann, 2000). All of these have led to strong demand for studying biological processes at the protein level, which is the main goal of proteomics. Therefore, high-throughput methods for the analysis of proteins are required (Kersten *et al.*, 2002; Kersten *et al.*, 2004a). Protein microarrays can be used for the global analysis of the expression, modification, molecular interaction, and function of proteins derived from specific cells, tissues or organisms (Zhu and Snyder, 2003).

Microarray technology started with the development of surfaces, detection methods, and applications for DNA microarrays. There are many differences between the generation of DNA microarrays and protein microarrays. DNA and proteins differ with regard to their chemical and physical characteristics. The molecular structure of the DNA is more uniform, the molecule is stable and consists of a hydrophilic, negatively charged phosphate backbone, whereas the structure of proteins is very diverse and each structure is quite unique. Furthermore, post-translational modifications influence the functions of the protein molecules. Apart from the simpler structure, fast and efficient amplification *via* **P**olymerase **C**hain **R**eaction (PCR) is a further advantage of DNA molecules. In contrast, for example, one of the biggest limiting factors for the generation of protein microarays is to produce as many different recombinant proteins as possible. This includes the high-throughput cloning of cDNAs and expression as well as purification in high-throughput format.

There are two main methods for high-throughput cloning of cDNAs:

1) The construction of ordered cDNA expression libraries (Bussow *et al.*, 1998, 2000; Clark *et al.*, 1999; T. Feilner, Berlin, congress proceedings[1]).
2) The directional high-throughput sub-cloning of open reading frames (ORFs) in recombination-based cloning vectors using gene specific primers (Heyman *et al.*, 1999; Walhout *et al.*, 2000).

For the expression of recombinant proteins in large-scale, heterologous expression systems are usually used. Preferred expression systems for the generation of recombinant proteins are *Escherichia coli* (Braun *et al.*, 2002; Bussow *et al.*, 1998; Hannig and Makrides, 1998), yeast (Cereghino and Cregg, 2000; Cregg *et al.*, 1993; Hamilton *et al.*, 2003; Zhu

*Address correspondence to this author at the Max Delbruck Center for Molecular Medicine, Department Neuroproteomics, Robert-Rossle-Str. 10, 13092 Berlin, Germany; Tel: + 49-30-9406-2636; Fax: + 49-30-9406-2629; E-mail: b.kersten@mdc-berlin.de

[1] Feilner, T., Kersten, B., Immink, R. G. H., Wehrmeyer, S., Gutjahr, C., Horn, S., Possling, A., Angenent, G. C. *et al.* (2002). Construction of an ordered Arabidopsis cDNA expression library for the generation of protein arrays and chips. *Proceedings of the 1st Plant-GEMs Meeting*, 29 September-2 October 2002, Berlin, Germany.

1570-1646/04 $45.00+.00

et al., 2001) and insect cell lines (Albala *et al.*, 2000). Braun and co-workers (2002) were able to purify 46% of 326 human proteins in a soluble form after expression in *E. coli*. However, it has been shown that only a part of the proteins can be expressed in soluble form in *E. coli* and purified under native conditions (Bussow *et al.*, 2004). Expression of proteins in eukaryotic systems, e.g. insect cells infected by baculovirus has the advantage of obtaining modified proteins (Albala *et al.*, 2000). Another critical point is that for functional studies, it must be ensured that the proteins are maintained in an active state, which means that the proteins have to be purified and immobilized onto the microarray under native conditions.

For the purification of hundreds of proteins in parallel, affinity tags such as glutathione S-transferase (GST)-, histidine (His)-, FLAG-tag and Protein A are widely used (Phizicky *et al.*, 2003; Scheich *et al.*, 2003). Purification using tags can be easily adapted to high-throughput format. The protocols have been optimized to ensure that the proteins are purified in a functional and biochemically active form.

Some of the tags have been compared by Braun and co-workers (2002) for high-throughput purification. They discovered that GST-fusion proteins have a larger success rate for expression and purification than His_6-tagged proteins under denaturing conditions. In contrast, Scheich and co-workers (2003) utilized an expression vector with a N-terminal tandem His_6- and GST-tag to compare the efficiency of high-throughput protein purification by means of nickel-nitriloacetic acid (Ni-NTA) agarose and glutathione agarose. Using tandem affinity tags, the purification is independent of different expression rates due to different tags. As documented by Scheich and co-workers (2003), the purification of proteins with Ni-NTA agarose is more efficient than the purification with glutathione agarose.

Another alternative strategy is the cell-free protein expression using *in vitro* transcription and translation systems. These methods simplify procedures for the expression and purification of proteins; furthermore, it is possible to express proteins, which could be toxic to the host (Allen and Miller, 1999).

2. SURFACES AND FORMATS

As mentioned earlier, in addition to the generation and isolation of appropriate capture molecules, the immobilization of the capture molecules and the surface of microarrays are important to keep the proteins in an active state. The surface of the protein microarrays has to be modified to achieve maximum binding capacity. The immobilization of the proteins is usually done by non-covalent or covalent attachments. Surfaces, which allow non-covalent attachment, can be positively charged (e.g. poly-L-lysine, aminosilane) or hydrophobic (e.g. nitrocellulose, polystyrene). Covalent attachments use a variety of chemically activated surfaces (e.g. aldehyde, epoxy, active esters) for the generation of protein microarrays. All the above-mentioned surfaces lead to a non-orientated (i.e. random) attachment of immobilized proteins.

An orientated immobilization requires specific molecules, e.g. Ni (Zhu *et al.*, 2001), nucleic acid (Weng *et al.*, 2002), biotin or streptavidin (Birkert *et al.*, 2000; Pavlickova *et al.*, 2002, 2003), which are coated to the surface and to which the proteins bind *via* respective specific tags. The oriented immobilization of proteins provides a better access to active binding sites. Peluso and co-workers (2003) compared random versus specific orientation of immobilized antibodies (capture molecules). They showed that the specific orientation of capture agents consistently increases the analyte-binding capacity of the surface in comparison with the randomly attached capture molecules.

There are different formats of protein microarrays. In this review three different formats will be described: (1) plain (non-gel coated) microarrays, (2) three dimensional microarrays, and (3) nanowells. A summary of other microarray formats is available in Glokler and Angenendt (2003).

1) Plain (non-gel coated) microarrays are treated with different chemicals, such as poly-L-lysine (Ge, 2000; Snapyan *et al.*, 2003), aldehyde (MacBeath and Schreiber, 2000) or epoxy derivatives (Angenendt *et al.*, 2003b).

2) Three dimensional microarrays have a gel-coated surface, such as polyacrylamide (Arenkov *et al.*, 2000) or agarose (Afanassiev *et al.*, 2000). This kind of microarrays includes FAST™ (fluorescence array surface technology slide) slides from Schleicher & Schuell (Dassel, Germany) coated with a nitrocellulose-derived polymer (Angenendt *et al.*, 2003b; Kersten *et al.*, 2003; Kukar *et al.*, 2002). Three dimensional microarrays have a higher immobilization capacity. The homogenous water environment minimizes protein denaturation. This could have positive influence on the active state of the proteins.

3) Nanowell microarrays contain miniature microwells with a diameter of 1 mm or less and a depth of approximately 300 μm. They were developed by Zhu and co-workers (2000) and are composed of standard glass slides with an open structure of polydimethylsiloxane (PDMS).

3. LABELING AND DETECTION

For detection, labeling of captured molecules is often used. This can be done by using fluorescence, radioactivity or chemiluminescense. The preferred labeling method is labeling with fluorescence dyes (e.g. Cy3 and Cy5), which is easy to handle, extremely sensitive and has a high detection resolution (Haab *et al.*, 2001). Detection of fluorescence labels is carried out using a laser scanner. There are both direct as well as indirect labeling methods. In the later case, proteins are tagged (e.g. with biotin) and can be screened by using a fluorescently labeled affinity reagent (e.g. streptavidin).

Another fluorescence-based detection method is the rolling circle amplification (RCA) (Schweitzer *et al.*, 2002). In this case, the detection antibody is tagged with a DNA-primer. The antibody-DNA-conjugate binds specifically to the immobilized interaction partner, which allows the following amplification *via* RCA. A circular DNA molecule hybridizes to the complementary primer and is amplified in presence of DNA polymerase and fluorescence-labeled nucleotides. The fluorescence, which can be measured at each spot afterwards, is directly proportional to the specific protein concentration in the original sample on the

microarray. This method has a high sensitivity, a wide dynamic range and excellent spot-to-spot reproducibility.

Sometimes the labeling of molecules can affect their activity. To avoid this, one can use non-labeling methods. The atomic force microscope (AFM) has been applied to detect arrays of immobilized antigenic rabbit IgG on gold. The IgG was covalently attached to substrates on the arrays by photolithography. After binding of goat anti-rabbit IgG antibodies, AFM was used to detect increases in the height of the antigen-antibody complexes (Jones *et al.*, 1998).

Another non-labeling method is the surface-enhanced laser desorption/ionisation (SELDI). This technology combines protein microarrays with time of flight mass spectrometry (MS-TOF). Starting with crude biological samples such as serum, cell lysates or other proteins, different protein subsets are extracted from the sample using different chromatographic chip surfaces. After removing unbound proteins, the immobilized proteins are identified by MS-TOF (reviewed in Tang *et al.*, 2004 and also see section 4.2.3).

Another technology, which should be mentioned in this review, is the surface plasmon resonance (SPR) (Houseman *et al.*, 2002). This technology is based on the measurement of the mass concentration of molecules on an activated gold chip, to which one of the interacting partners is attached. The other interacting partner flows over the attached molecules and leads to a change in the local mass concentration upon binding. However, the commercially available SPR chips are not suitable for high-throughput analysis at the moment.

Surfaces and labeling methods, which were applied in several molecular interaction studies, are sorted by application in Table 1. Detailed descriptions of the different interaction studies using protein and antibody arrays are described in the following section (section 4).

4. APPLICATION

There are mainly two types of protein microarrays: analytical and functional protein microarrays (Templin *et al.*, 2003; Zhu and Snyder, 2003).

Analytical protein microarrays (section 4.1) are used to profile antibodies or proteins and consist of high-density arrays of different capture molecules; proteins (antigens) (section 4.1.1) or antibodies/antibody mimics (section 4.1.2) (Haab *et al.*, 2001; Nielsen *et al.*, 2003; Robinson *et al.*, 2002).

Functional protein microarrays (section 4.2) are created by immobilizing large numbers of purified, recombinant, preferably native proteins from a given cell, meristem or organism on a solid surface. This is probably the most complex area of protein microarrays. These arrays can be used to screen the interaction of the immobilized proteins with other proteins, DNA, small molecules or the modification of the proteins by enzymes.

The analysis of protein-protein interactions was first demonstrated by Zhu and co-workers (2001) (section 4.2.1). Initial studies of protein-DNA interactions using microarrays were performed and may be used in future to identify and characterize nucleic acid-binding proteins, such as transcription factors (section 4.2.2). Another possibility is to detect the interactions of proteins with small molecules/ligands using protein microarrays (section 4.2.3). A further use of protein microarrays is to analyze the modification of proteins by enzymes, for example protein kinases (section 4.2.4).

4.1. Profiling Studies Using Analytical Protein Microarrays

Analytical microarrays used for antigen-antibody interactions can be divided in two categories, based on the component bound on the array surface, (1) protein/antigen arrays (section 4.1.1) and (2) antibody arrays (section 4.1.2). Although antibodies are proteins, we distinguish between each other, because of their different medical roles and their difference in detection modes. Antigen-antibody interactions on microarrays will be of enormous interest for medical diagnostics. For example, protein microarrrays are used for screening serum to detect antibodies produced due to infectious or autoimmune diseases. Diagnostic protein markers may be identified by screening of antibody arrays with serum (Cahill, 2001; Glokler and Angenendt, 2003; Petricoin *et al.*, 2002a, b; Walter *et al.*, 2002). For diagnostic applications of protein and antibody microarrays, the detection limits of the respective captured molecules in serum samples are crucial. In the case of protein microarrays, IgG serum-concentrations from natural immune responses are very important, which vary typically from 10 ng/ml (Anthony *et al.*, 1994) to 3 μg/ml (Granoff *et al.*, 1986). For antibody microarrays the detection limits have to be below threshold prognostic values, e.g. 5 μg/ml for the breast cancer markers carcinoembryonic antigen (CEA), 15 mg/ml for c-cerbB-2 and 15.3 mg/ml for cancer antigen (CA) (Molina *et al.*, 1998).

4.1.1. Protein/Antigen Arrays

Until now, protein/antigen arrays have been established by several groups, differing in surface materials, sensitivities and applications (reviewed in: Lopez and Pluskal, 2003; Seong and Choi, 2003; Templin *et al.*, 2003; Zhu and Snyder, 2003).

In 1998, protein macroarrays on nylon filters, a conventional protein blotting material, were established (Bussow *et al.*, 1998). Later polyvinylidene difluoride (PVDF), also a conventional protein support material, was used for a microarray containing 92 human proteins spotted on a microscopic glass slide (76 mm x 26 mm), with a theoretical spot density of up to 4,800 proteins/chip (~243 spots/cm^2) (Lueking *et al.*, 1999). In these early experiments high sensitivities (250 amol or 10 pg/spot) were reached by spotting the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and screening the microarray with a monoclonal anti-GAPDH-antibody. Using polyacrylamide, another conventional material, microarrays containing the same protein GAPDH, reached a calculated detection limit of 7.2 fmol or 288 pg/spot (Lueking *et al.*, 2003). Polyacrylamide was used previously for various applications of microarrays such as immunoassays, sandwich immunoassays or Enzyme-linked immunosorbent assay (ELISA) (Arenkov *et al.*, 2000). Later, materials used for DNA microarrays, were tested for protein microarrays. Using poly-L-lysine surfaces, detection limits of 20 pg/spot were reached (Haab *et al.*, 2001). A big

Table1. Summary of Surfaces and Detection Methods for Antibody and Protein Microarrays

Interaction of	Surface	Labeling of the detected molecule	References
Antibody-antigen	poly-L-lysine	protein-Cy3 or -Cy5	Haab *et al.*, 2001
	nitrocellulose	protein-L-HRP / ECL	deWildt *et al.*, 2000
	*FAST™	RFP	Kukar *et al.*, 2002
	aldehyde	RFP	Kukar *et al.*, 2002
	polyacrylamide	different proteins-FITC	Arenkov *et al.*, 2000
	streptavidine	IL8-protein-Alexa 546	Peluso *et al.*, 2003
	epoxy	fibrinogen-Cy3	Angenendt *et al.*, 2003b
	amine	fibrinogen-Cy3	Angenendt *et al.*, 2003b
Antigen-antibody	PVDF	antibody-HRP / colour	Lueking *et al.*, 1999
	nickel-coating	anti-GST-Cy5	Zhu *et al.*, 2001
	epoxy	anti-mouse-IgG-Cy5	Angenendt *et al.*, 2003b
	amine	anti-mouse-IgG-Cy5	Angenendt *et al.*, 2003b
	*FAST™	anti-mouse-IgG-Cy3	Kersten *et al.*, 2003
	polyacrylamide	anti-mouse-IgG-Cy3	Kersten *et al.*, 2003
	*FAST™	streptavidine-Cy3	Kim *et al.*, 2002
	*FAST™	anti-goat- or anti–mouse- or anti-rabbit-Cy5	Michaud *et al.*, 2003
	polyacrylamide	anti-human-IgG-Cy3	Lueking *et al.*, 2003
	polyacrylamide	anti-mouse-IgG-FITC	Arenkov, *et al.*, 2000
	poly-L-lysine	anti-Flag or anti-IgG-Cy3 or -Cy5	Haab *et al.*, 2001
	poly-L-lysine	anti-human-IgG-Cy3	Robinson *et al.*, 2002
Protein-protein	polyamine	anti-protein-Cy3 and -Cy5	Seitz, in prep.
	*FAST™	anti-protein-Cy3 and -Cy5	Seitz, in prep.
	*FAST™	anti-protein-Cy3 and -Cy5	Zeilinger and Seitz, in prep.
	aldehyde	protein-Alexa 488, -Cy3, -Cy5, -BODIPY-FL	MacBeath and Schreiber, 2000
	nickel-coating	streptavidine-Cy3	Zhu *et al.*, 2001
Protein-DNA	*FAST™	dsOligo-Cy3	Kersten *et al.*, 2004b
	nitrocellulose	DNA probe-IRDye 800	Snapyan *et al.*, 2003
Protein-small molecules	aldehyde	small molecule-Alexa 488, -Cy3, -Cy5	MacBeath and Schreiber, 2000
	nickel-coating	streptavidine-Cy3	Zhu *et al.*, 2001
Protein-enzymes	polyacrylamide	fluorescence or colour	Arenkov *et al.*, 2000
	BSA-NHS	[γ-^{33}ATP]	MacBeath and Schreiber, 2000
	PDMS	[γ-^{33}ATP]	Zhu *et al.*, 2000
	*FAST™	[γ-^{33}ATP]	Kramer *et al.*, 2004
	**Jerini pepSTAR™	[γ-^{32}ATP]	Lizcano *et al.*, 2002
	***APTES-derivatized	[γ-^{33}ATP]	Falsey *et al.*, 2001
	gold	SPR, antibody-AlexaFluor 532, [γ-^{32}ATP]	Houseman *et al.*, 2002
	avidin-derivatized	anti-phophoserine-FITC	Lesaicherre *et al.*, 2002
	avidin-derivatized	anti-phophotryosine antibodies-FITC	Lesaicherre *et al.*, 2002
	hydrogel	fluorescent dye for phosphoamino acids	Martin *et al.*, 2003

*: coated with nitrocellulose-derived polymer, **: coated with cellulose-derivatized, ***: 3-aminopropyl-triethoxysilane, HRP: horseradish peroxidase, PVDF: polyvinylidene difluoride, BSA-NHS: BSA-N-hydroxysuccinimide, PDMS: polydimethylsiloxane, ECL: enhanced chemiluminescence, RFP: red-fluorescent protein, FITC: fluorescein isothiocyanate, IRDye: near-infrared fluorescence dye, dsOligo: double stranded oligonucleotide, in prep.: in preparation. For rest of the abbreviations see list at the end of the article.

step forward was the first proteome microarray, containing 5,800 different purified yeast proteins (Zhu *et al.*, 2001). In the same study a new technical aspect was the use of two tags, one was used for purification (GST-tag), the other (His_6-tag) used for orientated immobilization of the proteins on a nickel-coated microarray.

As many as 1,152 features (196 distinct molecules in replications) containing microarray on poly-L-lysine coated microscope slides was reported for screening with sera from patients suffering from diverse autoimmune diseases (Robinson *et al.*, 2002). It contained 196 distinct molecules, including recombinant or purified proteins, nucleic acid-based antigens and different medically relevant peptides. Unlabelled human IgG- and IgM-antibodies were used for normalization and a mixture of antibodies pre-labeled with Cy-3 and Cy-5 assisted as orientation markers. In titration experiments on these protein microarrays, linear detection with varying antibody concentrations ranging from 1 to 900 ng/ml was demonstrated. Compared to ELISA, these authors demonstrated a 4 - 8 fold higher sensitivity of microarray technology.

In contrast to most studies focused on IgG or IgM induced protective immune responses, microarray technology can also be used for quantitative measurement of serum allergen-specific IgE. Therefore specific allergens spotted on FAST™ slides, reached a detection limit of 62 µg/ml (Kim *et al.*, 2002). For the study of allergies coupled to nutrients, nutrient specific protein microarrays have to be generated and screened with patient sera. As an initial step, we have generated one of the first plant protein microarrays (Kersten *et al.*, 2003). We were able to detect the immobilized tagged proteins using an anti-RGS-His_6-antibody with a sensitivity of 100 amol/spot on polyacrylamide or 2 fmol/spot on FAST™ slides, respectively. These high sensitivities were also achieved by using epoxy microarrays, with a detection limit of 100 amol/spot (Angenendt *et al.*, 2003b). Besides applications in screening of serum, the study of the cross-reactions of antibodies on whole proteome microarrays is an additional application for protein microarrays (Michaud *et al.*, 2003).

4.1.2. Antibody Arrays

In a proof-of-principle experiment for antibody arrays, macroarrays of nitrocellulose with a theoretical spot density up to 18,342 spots/529 cm^2 (~35 spots/cm^2) were used for screening immobilized recombinant scFvs-antibody fragments (single chain Fv-fragment, antibody variable region fragment) (de Wildt *et al.*, 2000). In a study in which 115 antibody/antigen pairs were tested on microarrays with poly-L-lysine coated slides, only 20% of arrayed antibodies provided specific and accurate signals (Haab *et al.*, 2001). The best sensitivities of 1 ng/ml were achieved for the antibody/antigen pairs of P38 delta, Numb and AIM-1 (Aurora and Ipl1-like Midbody associated protein).

The correct and reproducible labeling of proteins is a crucial step in the detection on antibody microarrays. To test the sensitivity of fluorescence detection independently from labeling, Kukar and co-workers (2002) tested RFP (red-fluorescent protein) on aldehyde or FAST™ slides containing anti-RFP antibodies. They reached a sensitivity of 1 µg/ml.

Although antibodies have a relatively common structural architecture, each antibody molecule has its own characteristic chemical properties. Therefore antibodies have to be tested on suitable surfaces to obtain optimal results with a given system (Angenendt *et al.*, 2002). Useful coatings are epoxy (dendrimer-arrays with several reactive groups) and amine with detection limits of 63 amol/spot (Angenendt *et al.*, 2003b). Orientated-binding of biotinylated antibodies or Fab-antibody fragments (monovalent antigen-binding antibody fragment) on streptavidin-coated microarrays resulted in increased sensitivity (Peluso *et al.*, 2003). This oriented-binding was achieved by N-linked glycosylation of the Fc portion of the IgGs, followed by oxidation and biotinylation; the disulfide bonds of Fab-antibody fragments were mildly reduced and biotinylated. In addition, porous silicon could also become an important surface due to a high homogeneity and a spot density up to 4,400 spots/cm^2 (Marko-Varga *et al.*, 2003).

In the studies described above, antibodies and antibody fragments were used for the generation of antibody microarrays. Besides antibody fragments, the so-called aptamers are especially interesting as antibody mimics for diagnostic approaches because of higher potential specificity and the low production costs. Aptamers derive from an *in vitro* evolution process called SELEX (systematic evolution of ligands by exponential enrichment) (Brody and Gold, 2000). With SELEX aptamer libraries of 30 – 40-mer random oligonucleotides, containing 10^{18} - 10^{24} individual sequences were screened against specific proteins. Eighty percent of the screened DNA/protein complexes have K_d values below 1 nM, comparable to most antigen-antibody complexes.

Further applications of analytical microarrays are possible using a new technology, called MIST (multiple spotting technique). MIST allows multiplex analysis on a single microarray thereby overcoming one major disadvantage of microarray technology. By spotting the second reactant in the same position, in which the first component was fixed to the surface, multiple separate tests are performed in parallel, using minimal volumes of analyte (Angenendt *et al.*, 2003a).

An example for alternative microarrays is a B-cell based sensor for the rapid identification of pathogens (Rider *et al.*, 2003). This sensor uses B-lymphocytes, which have been modified to emit light within seconds of exposure to specific pathogens. This system is very sensitive, as it can detect 50 colony-forming units (CFU) of *Yersinia pestis* specific cells in only 3 min. In an additional experiment, 500 CFU of the pathogenic *E. coli* strain O157:H7 per g lettuce were detected in only 5 min, compared to PCR, with 30 - 60 min for detection of 10 - 10,000 CFU. A comparable approach for a biosensor is based on measurements of changes in impedance using gold and platinum electrodes upon adsorption of recombinant antigens of a pathogen (Diniz *et al.*, 2003).

These last examples for new techniques are very promising for use as diagnostic tools, but their practicability has to be established in the future for a wide range of applications for protein microarrays. In conclusion, all presented approaches have fulfilled the criterion of sensitivity but only for selected probes. However, it is necessary to optimize systems with many probes present on one microarray, especially if they are of different kinds (antibodies, proteins, peptides, etc) as published by Robinson and co-workers (2002).

4.2. Interaction Studies Using Functional Protein Microarrays

4.2.1. Protein-Protein Interactions

Knowledge of the genomic sequences and transcriptional expression level do not allow for a reliable prediction of the function of proteins (Eisenberg *et al.*, 2000). By identifying the interaction between a protein with an unknown function and a protein with a known function, it is often possible to elucidate the function of the uncharacterized protein.

Currently, the most common and applied method to identify protein-protein interactions *in vivo* is the yeast two-hybrid system. This method has been applied to several organisms. Using yeast two-hybrid screens, proteins which physically interact with one another can be identified. The interacting proteins or protein domains are expressed either as fusion proteins with a Gal4 transcription-activation

domain (prey) or with a Gal4 DNA-binding domain (bait), respectively. Only if both proteins interact with each other, a reporter gene is transcribed. The biochemical activity of the reporter gene reflects the strength of the interaction.

Comprehensive research studies using the yeast two-hybrid system have been carried out (Fromont-Racine *et al.*, 1997; Hua *et al.*, 1998). Those studies have led to the identification and classification of protein networks and pathways. Two large-scale efforts for *Saccharomyces cerevisiae* were performed (Ito *et al.*, 2001; Uetz *et al.*, 2000). A comparison of the results shows that only 6% of the 2,200 reported interactions were found in both studies (von Mering *et al.*, 2002).

Using an affinity purification approach of proteins followed by MS-based protein identification, two studies were able to identify several protein complexes in *S. cerevisiae* (Gavin *et al.*, 2002; Ho *et al.*, 2002). For that approach, a gene-specific cassette containing a tandem affinity purification tag was inserted by a homologous recombination at the 3'end of the corresponding genes. After growing cells to mid-log phase, complexes were purified by using affinity purification and analyzed by MALDI-TOF MS. A comparison of the data obtained by these two MS-based approaches also showed a very weak overlapping between them. Ho and co-workers (2002) selected 725 bait proteins and Gavin and co-workers (2002) selected 1,739 baits for this affinity purification approach. Only 115 baits are common in both studies. Furthermore, only a little overlap between the yeast two-hybrid interactions and the protein complexes obtained by MS studies was observed by Bader and Hogue (2002). Data quality is important in the rapid identification of putative protein-protein interactions. At the moment, large-scale techniques do not show enough internal consistency to warrant complete acceptance of the resulting data. This indicates that each assay will have to be carried out multiple times before achieving high quality data (Bader and Hogue, 2002). In particular, time consuming and expensive methods like genome-wide yeast two-hybrid screens for eukaryotic organisms are difficult to repeat several times. In contrast to the yeast two-hybrid method, repetitions of the experiment and testing a variety of different experimental conditions can be done very easily by means of protein microarray technology.

Use of a whole proteome microarray for the functional characterization of proteins offers the possibility for a high-throughput analysis. In order to produce such arrays, it is necessary to clone, express and purify thousands of proteins. So far this has only been done for the relatively simple organism *S. cerevisiae* (Hudson *et al.*, 1997; Zhu *et al.*, 2001). Different strategies for the creation of large sets of non-redundant expression libraries from different organisms and tissues have been developed (Schweitzer *et al.*, 2003).

Recently, it has been shown that proteome-wide microarrays can be used to screen for protein-protein interactions (Zhu *et al.*, 2001). A total of 5,800 different affinity purified proteins were spotted in duplicate onto nickel-coated microscope slides and were screened for protein-protein interactions with biotinylated proteins. For the identified interactions it does not matter which protein of the interaction partners was immobilized onto the microarray and which protein was in solution (Zhu *et al.*, 2001). Again, several protein-protein interactions identified by the proteome-wide microarray technique were missing in the large-scale yeast two-hybrid and/or the affinity purification-MS studies and vice versa.

The strength of protein microarrays for the identification of protein interaction with respect to sensitivity and specificity was shown by identifying a single spot of the FKBP12-rapamycin-binding protein within an array of over 10,000 spots of another protein (MacBeath and Schreiber, 2000).

During the last years, the human proteome became more and more interesting and several national and international activities started with the goal of a systematic understanding[2]. A generation of a human proteome microarray with several thousand of different protein species including post-translational modifications of the immobilized proteins is not so far-off. Within the Human Brain Proteome Project, a microarray-based method was developed to identify protein interactions involved in neurodegenerative diseases (H. Seitz, manuscript in preparation). Proteins from a human expression library were expressed and purified under native and denaturing conditions in 96 well format. Immobilized proteins (native and denatured proteins) were screened for interaction partners. Native, purified and spotted proteins showed higher and specific interactions than the denatured ones. This means that it is possible to identify linear and conformational epitopes for protein-protein interaction. Using 384 different proteins, we have identified a number of highly specific protein-protein interactions under a variety of different experimental conditions (data not shown). While we have been able to verify already known protein interactions, several new protein-protein interactions have been identified. So far approximately 50% of the identified protein interactions were verified by SPR measurements (C. Zeilinger and H. Seitz, manuscript in preparation).

It can be concluded from results obtained by different groups that protein-protein interaction data from microarrays are highly reliable in terms of sensitivity and specificity. It is relatively straightforward to accomplish the systematic identification of stable protein-protein interactions, but detecting transient regulatory interactions is still difficult. It is important to develop computational systems that can integrate and visualize all available interactions identified with different methods. Taken together, protein microarrays are a powerful tool to study protein-protein interactions and are complementary to genome-wide *in vivo* screens.

4.2.2. Protein-DNA Interactions

Protein-DNA interactions drive basic cellular processes, such as transcription, replication, and recombination. The identification of such interactions and the determination of their specificity by measuring the effects of mutations on the interaction are important to understand these processes.

Direct methods, e.g. *in vivo* footprinting allow target identification for single DNA-binding proteins (Samitt *et al.*,

[2] www.hupo.org

1989). A genome-wide location of DNA-binding proteins is enabled by the ChIP-chip approach. This technology combines chromatin immunoprecipitation (ChIP) for the precipitation of protein-DNA complexes with cDNA microarray hybridization for the identification of the DNA-fragment, which was recognized by the analyzed protein. This method has been used to map transcriptional networks in yeast (Lee *et al.*, 2002). However, this method is limited by the need for antibodies against the DNA-binding proteins analyzed.

Several *in vitro* methods have been used for the analysis of protein-DNA interactions. Many of them only permit the analysis of one interaction at a time, such as gel mobility-shift analysis (Weigel *et al.*, 2002), south-western blotting (Cicchini *et al.*, 2002), ELISA-based methods (Kersten *et al.*, 2001), or Biacore analysis (SPR analysis) (Blaesing *et al.*, 2000; Kersten *et al.*, 2001). These methods are costly and limited by low-throughput (section 3).

Methods have been established, which allow the selection of several interaction partners for one target. Taken a given DNA-binding protein, *in vitro* selection has permitted the sampling of multiple DNA-binding sites (Jiang *et al.*, 2000). For given target DNA sites, phage display has emerged as a powerful tool to select, for example transcription factors that recognize the given target out of millions of protein variants (Cicchini *et al.*, 2002). However, such selection methods are time consuming and often provide only a partial view of the binding site specificity because only the strongest interactions are selected. This limitation has been overcome by the development of microarray-based methods for studying protein-DNA interactions allowing for the screening of hundreds or even thousands of defined targets at once in high-throughput fashion with a DNA or a protein of interest.

Bulyk and co-workers (1999) created dsDNA arrays by enzymatically converting ssDNA arrays. They applied DNA arrays to characterize sequence specific DNA recognition by zinc-finger proteins (Bulyk *et al.*, 2001).

The first attempts to apply the protein array technology to the analysis of protein-DNA interactions were made by Ge and co-workers (2000) using macroarrays generated by dot blotting of 48 different proteins including several transcription factors onto a nitrocellulose membrane. The array was probed with a ^{32}P-labelled dsDNA as well as ssDNA (64-mer oligonucleotides) containing the adenovirus major late core promotor elements. Despite the fact that many DNA-binding proteins bind dsDNA, the overall pattern of protein-ssDNA interactions detected was similar to that of protein-dsDNA interactions, suggesting that the tested DNA-binding proteins were capable of binding both. Snapyan and co-workers (2003) studied protein-DNA interactions, which govern the gene expression from *Bacillus stearothermophilus PargCo* promoter-operator region. They used DNA probes labeled with near-infrared fluorescence dyes (IRDye) and were able to detect 12.9 amol/spot of the cyclic adenosine monophosphate receptor protein (CRP) probed with a 76 bp DNA. In a recent study, we applied for the first time protein microarrays with a solid support (FAST™ slides) in combination with Cy5-fluorescence detection to analyze protein-DNA interactions (Kersten *et al.*, 2004b). As a model system, we chose the well-characterized interaction of the bacterial replication initiator DnaA with its cognate binding site, the DnaA box. UV crosslinking of the protein with the dsDNA probe and subsequent nanoLC-MALDI-TOF mass spectrometry located the site of the determined interaction.

In addition to dsDNA microarrays and the ChIP-chip technology, the application of protein microarray technology will help in future to reveal the molecular targets of DNA-binding proteins, such as transcription factors. In parallel experiments, it enables the simultaneous analysis of numerous DNA-binding proteins with respect to many DNA probes, which are applied to the microarray in small volumes.

In order to reveal multi-input motifs in transcriptional regulatory networks (Lee *et al.*, 2002), which are sets of regulators (transcription factors) that bind to a set of gene promotors, the application of protein microarrays may be very interesting, because it allows for the parallel selection of a set of transcription factors that recognize a given target DNA sequence in one hybridization. The inclusion of protein mutants into the microarray analysis (Kersten *et al.*, 2004b) may help to encircle amino acids or protein domains, which are important for the specific protein-DNA contact.

4.2.3. Protein-Small Molecule Interactions

In the fast growing field of chemical genomics, protein microarrays have been primarily developed to detect interactions between small molecules and proteins. Chemical genomics has been defined as genome wide study of biological processes by small molecule intervention (MacBeath, 2001). Besides metabolic reactions, this field includes each type of modulation of protein activities by non-covalent as well as the reversible covalent binding of small molecules as ligands to proteins. As proteins reflect the chemistry which takes place inside cells, alterations in protein chemistry resulting in physiological and/or pathological features came into the focus of the medical, pharmaceutical, toxicological and nutritional researchers.

Since the mid-nineties, the use of DNA microarrays has become a useful tool to study gene expression profiles in cells, tissues and organisms. Especially the analysis of expression patterns from genes of specific metabolic and regulatory networks are of enormous interest. These expression patterns may be influenced by the progression of multifactorial and complex diseases and by metabolic shifts resulting from different nutritional and environmental conditions. DNA microarrays have already been used to study cancer, inflammatory and cardiovascular diseases, diabetes and obesity, effects of high fat diets, nutrients, isoflavones and retardation of ageing by caloric restriction (Burton and McGhee, 2004; Haupl *et al.*, 2002; Liang and Pardee, 2003; Page *et al.*, 2003; Simon *et al.*, 2003).

Proteomic approaches such as SELDI-TOF MS (Reddy and Dalmasso, 2003) are rapidly finding many practical applications (Dare *et al.*, 2002; Dayal and Ertel, 2002; He *et al.*, 2003; Yip and Lomas, 2002). SELDI-TOF MS (section 3) allows study of the dynamics of cellular and tissue-specific "protein signatures" resulting from interaction with external small molecules as well as the identification of relevant biomarkers for disease characterization.

To study direct interaction of small molecules with proteins and to get binding profiles, the use of functional protein microarrays is gaining increasing interest. In their pioneer work, MacBeath and Schreiber (2000) tested some pre-conditions for applying protein microarrays to study protein-ligand interactions. Using model proteins, they demonstrated the functionality of proteins bound on coated glass slides and the possibility of detecting low affinity ligands with functional protein microarrays. For this, they attached the FKB12-rapamycin-binding domain (FRB) of the FKBP12-rapamycin-associated protein (FRAP) covalently to aldehyde coated glass slides. FRAP is a protein from the nutrient-sensitive targets of rapamycin proteins (TOR) signaling-network (Hardwick *et al.*, 1999). Fluorescence-labeled FKBP12 bound specifically to the immobilized FRB depending on the presence of the small molecule rapamycin. In a more direct assay format, specific ligand-binding reactions of an anti-digoxigenin-antibody, streptavidin and FKBP12 were reproduced on protein microarrays using ligands (a steroid derivative of digoxigenin, the vitamin biotin and four different ligands of FKBP12). The ligands were coupled with a flexible chemical linker to fluorescence-labeled bovine serum albumin (BSA). This type of labeling enabled the detection of interactions possessing dissociation constants from the nanomolar to the micromolar range (MacBeath and Schreiber, 2000).

In contrast to MacBeath and Schreiber (2000), in our proof-of-principle study we coupled the vitamin biotin with a flexible linker directly to Cy3 without BSA and were able to detect the interaction of biotin with streptavidin and an anti-biotin-antibody, which were immobilized on FAST™ slides (Fig. **1A**). To exclude non-specific binding of the linker, we incubated a microarray from the same series with the Cy3-linker (Fig. **1B**). Whereas the indirect labeling method with fluorescence-BSA (MacBeath and Schreiber, 2000) permits the detection of low affinity-interactions due to the multivalency of the BSA-conjugates, the direct labeling in our study may have the advantage to observe differences in affinity (MacBeath and Schreiber, 2000).

A practical genome-wide approach to determine new protein interactions using a proteome microarray was established by Zhu and co-workers (2001). Protein-lipid interactions were measured using five different types of biotinylated liposomal phosphoinositides (PI) as ligands. In these experiments, a total of 150 protein targets were identified out of 5,800 different proteins, and 35% of them correspond to uncharacterized proteins. Approximately half of the known identified protein targets were membrane-associated proteins, which are anticipated as denatured in this approach. Interestingly, some of the PI-binding proteins were well-known enzymes and protein kinases mainly involved in glucose metabolism, discovering new interrelations between the metabolic networks of lipids and carbohydrates.

Recent protein microarray studies focused on cellular, nucleic or membrane-associated receptors because they are mostly the targets of drugs and nutrients. These receptors are involved in signaling pathways, cell growth and apoptosis, transport processes, gene expression and metabolic shifts. Besides transcription factors and nucleic receptors, the **G** protein coupled receptors (GPCRs) are of special interest because they are the largest family of receptors and the largest class of drug targets (Neumann *et al.*, 2002) in the human genome. They mediate the majority of cellular responses to hormones, neurotransmitters, nutrients, flavors and other bioactive compounds. In this context, Neumann and co-workers (2002) published a simple method to immobilize functional, detergent-solubilized GPCRs on micro-

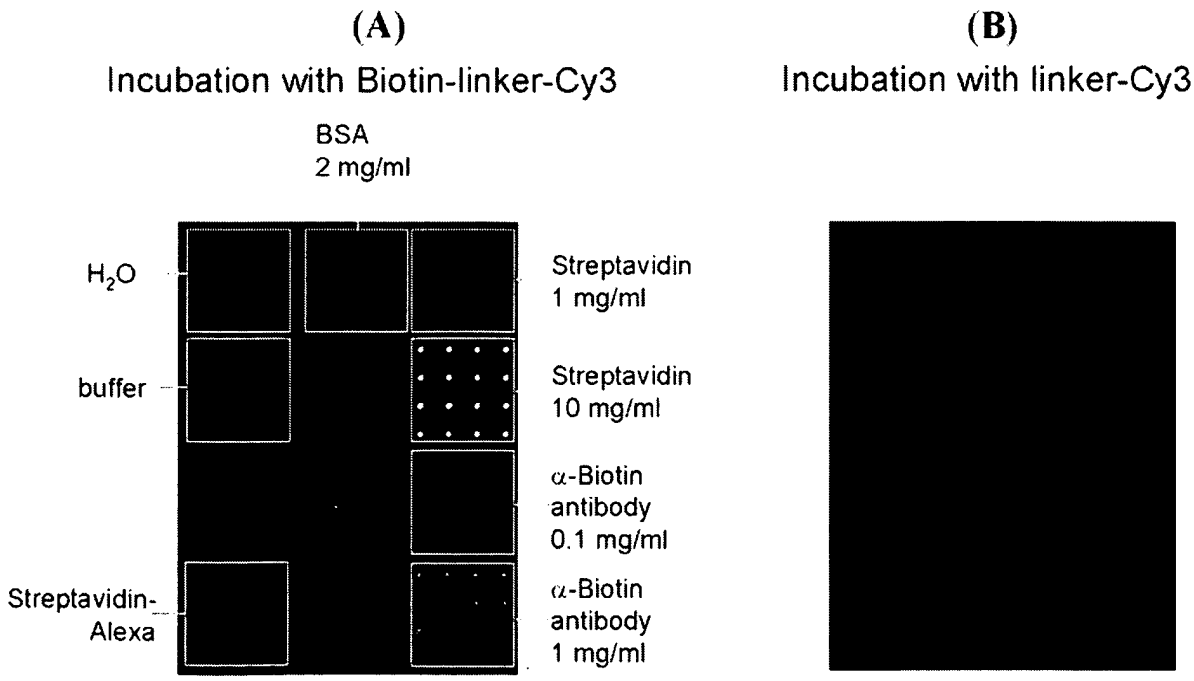

Fig. (1). Detecting targets of the small molecule biotin on FAST-slides. (A) Slide probed with biotin-linker-Cy3. (B) Slide probed with linker-Cy3. Protein microarrays were generated using a Q-array system (Genetix, New Milton, UK).

arrays. To study the binding behavior of GPCRs, Fang and co-workers (2002) developed a method for printing membrane preparations to gamma-aminopropylsilane (GAPS) surfaces. Specific binding of fluorescence-labeled neurotensin was demonstrated on microarrays containing different GPCRs. Subtype specific binding of a receptor antagonist analogue to one of the analyzed receptors was observed and could be inhibited with a second synthetic analogue with higher affinity. A recent overview of GPCR microarrays and their use in drug development strategies have been published by Fang and co-workers (2003).

Besides protein microarrays to analyze protein-small molecule interactions, microarrays based on natural or synthetic chemical compound libraries have been developed (Kimmich and Park, 2003; Knepper *et al.*, 2003; Kuruvilla *et al.*, 2002; MacBeath, 2001). An advantage of these compound arrays compared to protein arrays is that the small molecules do not have to be labeled. Therefore, it is possible to exclude the influence of labeling on binding specificities and affinities of small molecules.

Nevertheless, the discovery of regulatory networks of gene expression and signaling pathways determined by receptor/ligand interactions is a very interesting field for developing protein microarray-based screening assays, predominantly for drug designers, pharmacologists, toxicologists and likewise for nutritional biochemists (Bode and Dong, 2004; Im, 2003; Rowinsky, 2003; Shen and Brown, 2003; Shtil, 2001). A challenge is the design of proteome microarrays to perform a proteome-wide drug targeting and a proteome-wide screening of side effects of single drugs or nutrients.

4.2.4. Protein Microarray-Based Enzymatic Assays

Protein microarrays have been successfully used to monitor enzymatic reactions. Arenkov and co-workers (2000) immobilized enzymes like horseradish peroxidase, alkaline phosphatase and β-D-glucuronidase on microarrays and tested their activities with substrates yielding color or fluorescent precipitates during enzymatic reactions. In contrast to this study, other groups immobilized protein substrates on the microarrays and incubated them with different enzymes (MacBeath and Schreiber, 2000; Zhu *et al.*, 2000). This approach is promising because it allows for the examination of post-translational modifications in a parallel fashion. Modifications to protein structures play a crucial role in regulating protein activity. Only the reversible phosphorylation of proteins, which is one of the most important modifications, has been analyzed by using protein microarray technology so far.

The first experiments for measuring kinase activity on protein microarrays were carried out on microarrays coated with a BSA-NHS monolayer to which three different specific kinase substrates were covalently attached (MacBeath and Schreiber, 2000). Zhu and co-workers (2000) used protein microarrays bearing PDMS coated microwells in which 17 different protein substrates were covalently immobilized. Nearly all of the protein kinases from *S. cerevisae* were analyzed and many novel activities were found as well as 27 protein kinases with an unexpected tyrosine kinase activity.

In a recent study, we performed the first screen for potential target substrates of a kinase by using the protein microarray technology (Kramer *et al.*, 2004). We used FAST™ slides, which are coated with a nitrocellulose-derived polymer, to identify targets of barley casein kinase IIα among 768 recombinant barley proteins. We identified novel target proteins as well as known substrates of the analyzed kinase.

To detect the molecular basis for the substrate specificity of kinases, peptide chips have been used (Falsey *et al.*, 2001; Houseman *et al.*, 2002; Lesaicherre *et al.*, 2002; Lizcano *et al.*, 2002). Lizcano and co-workers (2002) analyzed a human serine/threonine kinase Nek6 by using peptide microarrays harboring >1,000 peptide species. They investigated a strong preference for a Leu, 3 residues N-terminal to the site of phosphorylation.

For the detection of phosphorylation events on protein microarrays different approaches have been applied. Radioactively labeled ATP ([γ^{32}-P]ATP or [γ^{33}-P]ATP) was used for the detection by different groups. MacBeath and Schreiber (2000) incubated the slides after the enzymatic reaction in a photoemulsion followed by microscopical analysis, whereas other groups used phosphorimager and/or X-ray-films for the detection of the radioactively labeled proteins/peptides (Falsey *et al.*, 2001; Houseman *et al.*, 2002; Lizcano *et al.*, 2002; Zhu *et al.*, 2000; Kramer *et al.*, 2004). Although the radioactivity-based detection method is the most sensitive one, it causes certain safety and disposal problems. Alternatively, immunodetection with antibodies against phosphorylated protein epitopes has been employed. Lesaicherre and co-workers (2002) used fluorescence-labeled anti-phosphoserine and anti-phosphotyrosine antibodies for the detection of phosphorylated peptides. Houseman and co-workers (2002) used an anti-phosphotyrosine-antibody with subsequent incubation of the chips with a fluorescence-labeled secondary antibody. Furthermore they applied SPR to analyze the binding of the anti-phosphotyrosine-antibody. They used immobilized 9-mer peptide substrates arrayed at high density on gold-coated glass surface.

Methods for the detection of phosphorylation events, which are based on phosphate-specific staining with fluorescent dyes, have been developed by Gast and co-workers (1999) and Martin and co-workers (2003). The advantage of fluorescence-based detection method lies in the possibility of using conventional array scanners for the evaluation of kinase assays. However this method still has to be developed further and cannot yet compete with the radioactive-based detection.

In the future, microarrrays with immobilized proteins or peptides can be used to analyze other modifications such as acetylation and methylation of proteins as well.

A completely different chip-based approach for the analysis of enzymatic reactions lies in the application of microfluidic chips. These chips contain microwells that are connected by tubing and allow liquid handling in a miniaturized format. Electrokinetic effects control the flow of liquid in these capillary systems. Thus, they represent miniaturized analytical devices ("labs-on-chips"concept; Bilitewski *et al.*, 2003). Hadd and co-workers (1997) performed an enzymatic

assay applying β-galactosidase and resorufin beta-D-galactopyranoside (RBG). RBG is a substrate of the β-galactosidase that is hydrolyzed to resorufin, a fluorescent product, which was detected during the assay. Using electrokinetic flow, precise concentrations of all reagents were mixed, and Michaelis-Menten constants were derived with and without an inhibitor added. Cohen and co-workers (1999) were able to monitor the phosphorylation of the kemptide peptide (heptapeptide LRRASLG, kinase substrate) by protein kinase A. They used electroosmosis for the transport of reagents within the network of a microfluidic chip. Moreover they were able to prepare on-chip dilutions of the reagents and measured kinetic constants of the reactions. Fluorescence-labeled peptides were used for the kinase assay. Since, phosphorylated peptides are negatively charged, compared with the non-phosphorylated form, substrates and products were separated by a subsequent electrophoresis step on chip. By this means the reaction products were detected and quantified. The possibility to determine enzymatic reactions by means of amperometric detectors on microfluidic chips was shown by Wang and co-workers (2003).

CONCLUSIONS

In recent years, protein and antibody microarray technology has become an important proteomic tool. This technology has several useful applications, such as screening of serum. The availability of the full human genome sequence will advance its application in analytical and functional studies of the human proteome. In this respect, the challenge is the creation of large sets of recombinant proteins in a preferably native confirmation, which is required for functional studies. A further issue is to retain the stability of proteins/antibodies after immobilization. Improvements in generating large sets of antibody reagents as well as expression of different proteins from a variety of host cells will promote the protein microarray technology in future.

ACKNOWLEDGEMENTS

We gratefully thank Jasmin Bastian and Meredith Hughes for reading the manuscript.

ABBREVIATIONS

AFM	=	Atomic force microscope
AIM	=	Aurora and Ip11-like Midbody associated protein
BSA	=	Bovine serum albumin
BSA-NHS	=	BSA-*N*-hydroxysuccinimide
CA	=	Cancer antigen
CEA	=	Carcinoembryonic antigen
Cfu	=	Colony-forming units
ChIP	=	Chromatin immunoprecipitation
CRP	=	Cyclic adenosine monophosphate receptor
Cy3	=	Indocarbocyanine
Cy5	=	Indodicarbocyanine
ELISA	=	Enzyme-linked immunosorbent assay
Fab-antibody fragment	=	Monovalent antigen-binding antibody fragment
FAST™ slide	=	Fluorescence array surface technology slide (Schleicher & Schuell)
FRB	=	FKB12-rapamycin-binding domain
FRAP	=	FKBP12-rapamycin-associated protein
GAP	=	Gamma-aminopropylsilane
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	=	G protein coupled receptors
GST	=	Glutathione S-transferase
IRdye	=	Infrared fluorescence dye
LC	=	Liquid chromatography
MALDI-TOF	=	Matrix assisted laser desorption/ionization-time of Flight
MIST	=	Multiple spotting technique
MS	=	Mass spectrometry
MS-TOF	=	Mass spectrometry-time of Flight
NTA	=	Nitriloacetic acid
ORF	=	Open reading frame
PCR	=	Polymerase chain reaction
PDMS	=	Polydimethylsiloxane
PI	=	Liposomal phosphoinositides
PVDF	=	Polyvinylidene difluoride
RBG	=	Resorufin beta-D-galactopyranoside
RCA	=	Rolling circle amplification
RFP	=	Red fluorescent protein
RGS	=	Arginine-glycine-serine
ScFv	=	Single chain Fv-fragment, antibody variable region fragment
SELDI	=	Surface-enhanced laser desorption/ionisation
SELEX	=	Systematic evolution of ligands by exponential enrichment
SPR	=	Surface plasmon resonance
TOR	=	Target of rapamycin proteins

REFERENCES

Afanassiev, V., Hanemann, V. and Wolfl, S. (2000). Preparation of DNA and protein micro arrays on glass slides coated with an agarose film. *Nucleic Acids Res.* **28:** E66.

Albala, J. S., Franke, K., McConnell, I. R., Pak, K. L., Folta, P. A., Rubinfeld, B., Davies, A. H., Lennon, G. G. *et al.* (2000). From genes to proteins: high-throughput expression and purification of the human proteome. *J. Cell Biochem.* **80:** 187-91.

Allen, S. V. and Miller, E. S. (1999). RNA-binding properties of *in vitro* expressed histidine-tagged RB69 RegA translational repressor protein. *Anal. Biochem.* **269:** 32-7.

Angenendt, P., Glokler, J., Murphy, D., Lehrach, H. and Cahill, D. J. (2002). Toward optimized antibody microarrays: a comparison of current microarray support materials. *Anal. Biochem.* **309:** 253-60.

Angenendt, P., Glokler, J., Konthur, Z., Lehrach, H. and Cahill, D. J. (2003a). 3D protein microarrays: performing multiplex immunoassays on a single chip. *Anal. Chem.* **75:** 4368-72.

Angenendt, P., Glokler, J., Sobek, J., Lehrach, H. and Cahill, D. J. (2003b). Next generation of protein microarray support materials: evaluation for protein and antibody microarray applications. *J. Chromatogr. A.* **1009:** 97-104.

Anthony, B. F., Concepcion, I. E., Concepcion, N. F., Vadheim, C. M. and Tiwari, J. (1994). Relation between maternal age and serum concentration of IgG antibody to type III group B streptococci. *J. Infect. Dis.* **170:** 717-20.

Arenkov, P., Kukhtin, A., Gemmell, A., Voloshchuk, S., Chupeeva, V. and Mirzabekov, A. (2000). Protein microchips: use for immunoassay and enzymatic reactions. *Anal. Biochem.* **278:** 123-31.

Bader, G. D. and Hogue, C. W. (2002). Analyzing yeast protein-protein interaction data obtained from different sources. *Nat. Biotechnol.* **20:** 991-7.

Bilitewski, U., Genrich, M., Kadow, S. and Mersal, G. (2003). Biochemical analysis with microfluidic systems. *Anal. Bioanal. Chem.* **377:** 556-69.

Birkert, O., Haake, H. M., Schutz, A., Mack, J., Brecht, A., Jung, G. and Gauglitz, G. (2000). A streptavidin surface on planar glass substrates for the detection of biomolecular interaction. *Anal. Biochem.* **282:** 200-8.

Blaesing, F., Weigel, C., Welzeck, M. and Messer, W. (2000). Analysis of the DNA-binding domain of Escherichia coli DnaA protein. *Mol. Microbiol.* **36:** 557-69.

Bode, A. M. and Dong, Z. (2004). Cancer prevention by food factors through targeting signal transduction pathways. *Nutrition* **20:** 89-94.

Braun, P., Hu, Y., Shen, B., Halleck, A., Koundinya, M., Harlow, E. and LaBaer, J. (2002). Proteome-scale purification of human proteins from bacteria. *Proc. Natl. Acad. Sci. U S A* **99:** 2654-9.

Brody, E. N. and Gold, L. (2000). Aptamers as therapeutic and diagnostic agents. *J. Biotechnol.* **74:** 5-13.

Bulyk, M. L., Gentalen, E., Lockhart, D. J. and Church, G. M. (1999). Quantifying DNA-protein interactions by double-stranded DNA arrays. *Nature Biotech.* **17:** 573-7.

Bulyk, M. L., Huang, X., Choo, Y. and Church, G. M. (2001). Exploring the DNA-binding specificities of zinc fingers with DNA microarrays. *Proc. Natl. Acad. Sci. U S A* **98:** 7158-63.

Burton, G. R. and McGehee, R. E. (2004). Identification of candidate genes involved in the regulation of adipocyte differentiation using microarray-based gene expression profiling. *Nutrition* **20:** 109-14.

Bussow, K., Cahill, D., Nietfeld, W., Bancroft, D., Scherzinger, E., Lehrach, H. and Walter, G. (1998). A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. *Nucleic Acids Res.* **26:** 5007-8.

Bussow, K., Nordhoff, E., Lubbert, C., Lehrach, H. and Walter, G. (2000). A human cDNA library for high-throughput protein expression screening. *Genomics* **65:** 1-8.

Bussow, K., Quedenau, C., Sievert, V., Tischer, J., Scheich, C., Seitz, H., Hieke, B., Niesen, F.H. *et al.* (2004). A catalogue of human cDNA expression clones and its application to structural genomics. *Genome Biol.* **5:** R71.

Cahill, D. J. (2001). Protein and antibody arrays and their medical applications. *J. Immunol. Methods* **250:** 81-91.

Cereghino, J. L. and Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiol. Rev.* **24:** 45-66.

Cicchini, C., Ansuini, H., Amicone, L., Alonzi, T., Nicosia, A., Cortese, R., Tripodi, M. and Luzzago, A. (2002). Searching for DNA-protein interactions by lambda phage display. *J. Mol. Biol.* **322:** 697-706.

Clark, M. D., Panopoulou, G. D., Cahill, D. J., Büssow, K. and Lehrach, H. (1999). Construction and analysis of arrayed cDNA libraries [Review]. *Methods Enzymol.* **303:** 205-233.

Cohen, C. B., Chin-Dixon, E., Jeong, S. and Nikiforov, T. T. (1999). A microchip-based enzyme assay for protein kinase A. *Anal. Biochem.* **273:** 89-97.

Cregg, J. M., Vedvick, T. S. and Raschke, W. C. (1993). Recent advances in the expression of foreign genes in Pichia pastoris. *Biotechnology (N Y)* **11:** 905-10.

Dare, T. O., Davies, H. A., Turton, J. A., Lomas, L., Williams, T. C. and York, M. J. (2002). Application of surface-enhanced laser desorption/ionization technology to the detection and identification of urinary parvalbumin-alpha: a biomarker of compound-induced skeletal muscle toxicity in the rat. *Electrophoresis* **23:** 3241-51.

Dayal, B. and Ertel, N. H. (2002). ProteinChip technology: a new and facile method for the identification and measurement of high-density lipoproteins apoA-I and apoA-II and their glycosylated products in patients with diabetes and cardiovascular disease. *J. Proteome Res.* **1:** 375-80.

de Wildt, R. M., Mundy, C. R., Gorick, B. D. and Tomlinson, I. M. (2000). Antibody arrays for high-throughput screening of antibody-antigen interactions. [comment]. *Nature Biotech.* **18:** 989-94.

Diniz, F. B., Ueta, R. R., Pedrosa, A. M., Areias Mda, C., Pereira, V. R., Silva, E. D., da Silva, J. G., Jr., Ferreira, A. G. *et al.* (2003). Impedimetric evaluation for diagnosis of Chagas' disease: antigen-antibody interactions on metallic electrodes. *Biosens. Bioelectron.* **19:** 79-84.

Eisenberg, D., Marcotte, E. M., Xenarios, I. and Yeates, T. O. (2000). Protein function in the post-genomic era. *Nature* **405:** 823-6.

Falsey, J. R., Renil, M., Park, S., Li, S. and Lam, K. S. (2001). Peptide and small molecule microarray for high throughput cell adhesion and functional assays. *Bioconjug. Chem.* **12:** 346-53.

Fang, Y., Frutos, A. G. and Lahiri, J. (2002). Membrane protein microarrays. *J. Am. Chem. Soc.* **124:** 2394-5.

Fang, Y., Lahiri, J. and Picard, L. (2003). G protein-coupled receptor microarrays for drug discovery. *Drug Discov. Today* **8:** 755-61.

Fromont-Racine, M., Rain, J. C. and Legrain, P. (1997). Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. *Nat. Genet.* **16:** 277-82.

Gast, R., Glokler, J., Hoxter, M., Kiess, M., Frank, R. and Tegge, W. (1999). Method for determining protein kinase substrate specificities by the phosphorylation of peptide libraries on beads, phosphate-specific staining, automated sorting, and sequencing. *Anal. Biochem.* **276:** 227-41.

Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M. *et al.* (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415:** 141-7.

Ge, H. (2000). UPA, a universal protein array system for quantitative detection of protein-protein, protein-DNA, protein-RNA and protein-ligand interactions. *Nucleic Acids Res.* **28:** e3.

Glokler, J. and Angenendt, P. (2003). Protein and antibody microarray technology. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **797:** 229-40.

Granoff, D. M., Shackelford, P. G., Suarez, B. K., Nahm, M. H., Cates, K. L., Murphy, T. V., Karasic, R., Osterholm, M. T. *et al.* (1986). Hemophilus influenzae type B disease in children vaccinated with type B polysaccharide vaccine. *New England J. Med.* **315:** 1584-90.

Haab, B. B., Dunham, M. J. and Brown, P. O. (2001). Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* 2: RESEARCH0004.

Hadd, A. G., Raymond, D. E., Halliwell, J. W., Jacobson, S. C. and Ramsey, J. M. (1997). Microchip device for performing enzyme assays. *Anal. Chem.* **69:** 3407-12.

Hamilton, S. R., Bobrowicz, P., Bobrowicz, B., Davidson, R. C., Li, H., Mitchell, T., Nett, J. H., Rausch, S. *et al.* (2003). Production of complex human glycoproteins in yeast. *Science* **301:** 1244-6.

Hannig, G. and Makrides, S. C. (1998). Strategies for optimizing heterologous protein expression in Escherichia coli. *Trends Biotechnol.* **16:** 54-60.

Hardwick, J. S., Kuruvilla, F. G., Tong, J. K., Shamji, A. F. and Schreiber, S. L. (1999). Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc. Natl. Acad. Sci. U S A* **96:** 14866-70.

Haupl, T., Burmester, G. R. and Stuhlmuller, B. (2002). New aspects of molecular biology diagnosis. Array technology and expression profile for characterization of rheumatic diseases. *Z. Rheumatol.* **61:** 396-404.

He, Q. Y., Yip, T. T., Li, M. and Chiu, J. F. (2003). Proteomic analyses of arsenic-induced cell transformation with SELDI-TOF ProteinChip technology. *J. Cell Biochem.* **88:** 1-8.

Heyman, J. A., Cornthwaite, J., Foncerrada, L., Gilmore, J. R., Gontang, E., Hartman, K. J., Hernandez, C. L., Hood, R. *et al.* (1999). Genome-scale cloning and expression of individual open reading frames using topoisomerase I-mediated ligation. *Genome Res.* **9:** 383-392.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P. *et al.* (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. *Nature* **415:** 180-3.

Houseman, B. T., Huh, J. H., Kron, S. J. and Mrksich, M. (2002). Peptide chips for the quantitative evaluation of protein kinase activity. *Nat. Biotechnol.* **20:** 270-4.

Hua, S. B., Luo, Y., Qiu, M., Chan, E., Zhou, H. and Zhu, L. (1998). Construction of a modular yeast two-hybrid cDNA library from human EST clones for the human genome protein linkage map. *Gene* **215:** 143-52.

Hudson, J. R., Jr., Dawson, E. P., Rushing, K. L., Jackson, C. H., Lockshon, D., Conover, D., Lanciault, C., Harris, J. R. *et al.* (1997). The complete set of predicted genes from Saccharomyces cerevisiae in a readily usable form. *Genome Res.* **7:** 1169-73.

Im, D. S. (2004). Discovery of new G protein-coupled receptors for lipid mediators. *J. Lipid Res.* **45:** 410-8.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. and Sakaki, Y. (2001). A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl. Acad. Sci. U S A* **98:** 4569-74.

Jiang, F., Wisen, S., Widersten, M., Bergman, B. and Mannervik, B. (2000). Examination of the transcription factor NtcA-binding motif by *in vitro* selection of DNA sequences from a random library. *J. Mol.Biol.* **301:** 783-93.

Jones, V. W., Kenseth, J. R., Porter, M. D., Mosher, C. L. and Henderson, E. (1998). Microminiaturized immunoassays using atomic force microscopy and compositionally patterned antigen arrays. *Anal. Chem.* **70:** 1233-41.

Kersten, B., Niemann, B. and Jahn, S. (2001). Development of single-chain Fv fragments from a human anti-double-stranded DNA antibody to study the influence of somatic mutations on antigen binding. *Exp. Clin. Immunogenet.* **18:** 96-9.

Kersten, B., Burkle, L., Kuhn, E. J., Giavalisco, P., Konthur, Z., Lueking, A., Walter, G., Eickhoff, H. *et al.* (2002). Large-scale plant proteomics. *Plant Mol. Biol.* **48:** 133-41.

Kersten, B., Feilner, T., Kramer, A., Wehrmeyer, S., Possling, A., Witt, I., Zanor, M. I., Stracke, R. *et al.* (2003). Generation of Arabidopsis protein chips for antibody and serum screening. *Plant Mol. Biol.* **52:** 999-1010.

Kersten, B., Feilner, T., Angenendt, P., Giavalisco, P, Brenner, W. and Bürkle, L. (2004a). Proteomic approaches in plant biology. *Current Proteomics* **1:** 131-44.

Kersten, B., Possling, A., Blaesing, F., Mirgorodskaya, E., Gobom, J. and Seitz, H. (2004b). Protein microarray technology and UV-crosslinking combined with mass spectrometry for the analysis of protein-DNA interactions. *Anal. Biochem.* **331:** 303-13.

Kim, T. E., Park, S. W., Cho, N. Y., Choi, S. Y., Yong, T. S., Nahm, B. H., Lee, S. and Noh, G. (2002). Quantitative measurement of serum allergen-specific IgE on protein chip. *Exp. Mol. Med.* **34:** 152-8.

Kimmich, R. D. and Park, W. K. (2003). Chemical libraries towards protein kinase inhibitors. *Comb. Chem. High Throughput Screen* **6:** 661-72.

Knepper, K., Gil, C. and Brase, S. (2003). Natural product-like and other biologically active heterocyclic libraries using solid-phase techniques in the post-genomic era. *Comb. Chem. High Throughput Screen* **6:** 673-91.

Kramer, A., Feilner, T., Possling, A., Radchuk, V., Weschke, W., Bürkle, L. and Kersten, B. (2003). Identification of barley CK2α targets by using the protein microarray technology. *Phytochemistry* **65:** 1777-84.

Kukar, T., Eckenrode, S., Gu, Y., Lian, W., Megginson, M., She, J. X. and Wu, D. (2002). Protein microarrays to detect protein-protein interactions using red and green fluorescent proteins. *Analy. Biochem.* **306:** 50-4.

Kuruvilla, F. G., Shamji, A. F., Sternson, S. M., Hergenrother, P. J. and Schreiber, S. L. (2002). Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays. *Nature* **416:** 653-7.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K. *et al.* (2001). Initial sequencing and analysis of the human genome. *Nature* **409:** 860-921.

Laurell, T. and Marko-Varga, G. (2002). Miniaturisation is mandatory unravelling the human proteome. *Proteomics* **2:** 345-51.

Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., Hannett, N. M., Harbison, C. T. *et al.* (2002). Transcriptional regulatory networks in Saccharomyces cerevisiae. *Science* **298:** 799-804.

Lesaicherre, M. L., Uttamchandani, M., Chen, G. Y. and Yao, S. Q. (2002). Antibody-based fluorescence detection of kinase activity on a peptide array. *Bioorg. Med. Chem. Lett.* **12:** 2085-8.

Liang, P. and Pardee, A. B. (2003). Analysing differential gene expression in cancer. *Nat. Rev. Cancer* **3:** 869-76.

Lizcano, J. M., Deak, M., Morrice, N., Kieloch, A., Hastie, C. J., Dong, L., Schutkowski, M., Reimer, U. *et al.* (2002). Molecular basis for the substrate specificity of NIMA-related kinase-6 (NEK6). Evidence that NEK6 does not phosphorylate the hydrophobic motif of ribosomal S6 protein kinase and serum- and glucocorticoid-induced protein kinase *in vivo*. *J. Biol. Chem.* **277:** 27839-49.

Lopez, M. F. and Pluskal, M. G. (2003). Protein micro- and macroarrays: digitizing the proteome. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **787:** 19-27.

Lueking, A., Horn, M., Eickhoff, H., Bussow, K., Lehrach, H. and Walter, G. (1999). Protein microarrays for gene expression and antibody screening. *Anal. Biochem.* **270:** 103-11.

Lueking, A., Possling, A., Huber, O., Beveridge, A., Horn, M., Eickhoff, H., Schuchardt, J., Lehrach, H. *et al.* (2003). A Nonredundant Human Protein Chip for Antibody Screening and Serum Profiling. *Mol. Cell Proteomics* **2:** 1342-1349.

MacBeath, G. (2001). Chemical genomics: what will it take and who gets to play? *Genome Biol.* **2:** COMMENT2005.

MacBeath, G. and Schreiber, S. L. (2000). Printing proteins as microarrays for high-throughput function determination. *Science* **289:** 1760-3.

Marko-Varga, G., Nilsson, J. and Laurell, T. (2003). New directions of miniaturization within the proteomics research area. *Electrophoresis* **24:** 3521-32.

Martin, K., Steinberg, T. H., Goodman, T., Schulenberg, B., Kilgore, J. A., Gee, K. R., Beechem, J. M. and Patton, W. F. (2003). Strategies and solid-phase formats for the analysis of protein and peptide phosphorylation employing a novel fluorescent phosphorylation sensor dye. *Comb. Chem. High Throughput Screen* **6:** 331-9.

McCarthy, J. E. (1998). Posttranscriptional control of gene expression in yeast. *Microbiol. Mol. Biol. Rev.* **62:** 1492-553.

Meri, S. and Baumann, M. (2001). Proteomics: posttranslational modifications, immune responses and current analytical tools. *Biomol. Eng.* **18:** 213-20.

Michaud, G. A., Salcius, M., Zhou, F., Bangham, R., Bonin, J., Guo, H., Snyder, M., Predki, P. F. *et al.* (2003). Analyzing antibody specificity with whole proteome microarrays. *Nat. Biotechnol.* **21:** 1509-12.

Molina, R., Jo, J., Filella, X., Zanon, G., Pahisa, J., Mu noz, M., Farrus, B., Latre, M. L. *et al.* (1998). c-erbB-2 oncoprotein, CEA, and CA 15.3 in patients with breast cancer: prognostic value. *Breast Cancer Res. Treat.* **51:** 109-19.

Neumann, L., Wohland, T., Whelan, R. J., Zare, R. N. and Kobilka, B. K. (2002). Functional immobilization of a ligand-activated G-protein-coupled receptor. *Chembiochem.* **3:** 993-8.

Nielsen, U. B., Cardone, M. H., Sinskey, A. J., MacBeath, G. and Sorger, P. K. (2003). Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc. Natl. Acad. Sci. U S A* **100:** 9330-5.

Page, G. P., Edwards, J. W., Barnes, S., Weindruch, R. and Allison, D. B. (2003). A design and statistical perspective on microarray gene expression studies in nutrition: the need for playful creativity and scientific hard-mindedness. *Nutrition* **19:** 997-1000.

Pandey, A. and Mann, M. (2000). Proteomics to study genes and genomes. *Nature* **405:** 837-46.

Parekh, R. B. and Rohlff, C. (1997). Post-translational modification of proteins and the discovery of new medicine. *Curr. Opin. Biotechnol.* **8:** 718-23.

Pavlickova, P., Jensen, N. M., Paul, H., Schaeferling, M., Giammasi, C., Kruschina, M., Du, W. D., Theisen, M. *et al.* (2002). Antibody detection in human serum using a versatile protein chip platform constructed by applying nanoscale self-assembled architectures on gold. *J. Proteome Res.* **1:** 227-31.

Pavlickova, P., Knappik, A., Kambhampati, D., Ortigao, F. and Hug, H. (2003). Microarray of recombinant antibodies using a streptavidin sensor surface self-assembled onto a gold layer. *Biotechniques* **34:** 124-30.

Peluso, P., Wilson, D. S., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., Heidecker, B., Poindexter, K. *et al.* (2003). Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal. Biochem.* **312:** 113-24.

Petricoin, E. F., 3rd, Hackett, J. L., Lesko, L. J., Puri, R. K., Gutman, S. I., Chumakov, K., Woodcock, J., Feigal, D. W., Jr. *et al.* (2002a). Medical

applications of microarray technologies: a regulatory science perspective. *Nature Genetics* **32** Suppl: 474-9.

Petricoin, E. F., Zoon, K. C., Kohn, E. C., Barrett, J. C. and Liotta, L. A. (2002b). Clinical proteomics: translating benchside promise into bedside reality. *Nature Reviews. Drug Discovery* **1:** 683-95.

Phizicky, E., Bastiaens, P. I., Zhu, H., Snyder, M. and Fields, S. (2003). Protein analysis on a proteomic scale. *Nature* **422:** 208-15.

Reddy, G. and Dalmasso, E. A. (2003). SELDI ProteinChip(R) Array Technology: Protein-Based Predictive Medicine and Drug Discovery Applications. *J. Biomed. Biotechnol.* **2003:** 237-241.

Rider, T. H., Petrovick, M. S., Nargi, F. E., Harper, J. D., Schwoebel, E. D., Mathews, R. H., Blanchard, D. J., Bortolin, L. T. *et al.* (2003). A B cell-based sensor for rapid identification of pathogens. *Science* **301:** 213-5.

Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B., Kamachi, M., Dean, E. J., Fournel, S., Fong, D. *et al.* (2002). Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nature Medicine* **8:** 295-301.

Rowinsky, E. K. (2003). Signal events: Cell signal transduction and its inhibition in cancer. *Oncologist* **8:** 5-17.

Samitt, C. E., Hansen, F. G., Miller, J. F. and Schaechter, M. (1989). *In vivo* studies of DnaA binding to the origin of replication of Escherichia coli. *EMBO J.* **8:** 989-93.

Scheich, C., Sievert, V. and Bussow, K. (2003). An automated method for high-throughput protein purification applied to a comparison of His-tag and GST-tag affinity chromatography. *BMC Biotechnol.* **3:** 12.

Schweitzer, B., Roberts, S., Grimwade, B., Shao, W., Wang, M., Fu, Q., Shu, Q., Laroche, I. *et al.* (2002). Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nature Biotech.* **20:** 359-65.

Schweitzer, B., Predki, P. and Snyder, M. (2003). Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics* **3:** 2190-9.

Seong, S. Y. and Choi, C. Y. (2003). Current status of protein chip development in terms of fabrication and application. *Proteomics* **3:** 2176-89.

Shen, Q. and Brown, P. H. (2003). Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways. *J. Mammary Gland Biol. Neoplasia* **8:** 45-73.

Shtil, A. A. (2001). Signal transduction pathways and transcriptional mechanisms as targets for prevention of emergence of multidrug resistance in human cancer cells. *Curr. Drug Targets* **2:** 57-77.

Simon, R., Mirlacher, M. and Sauter, G. (2003). Tissue microarrays in cancer diagnosis. *Expert Rev. Mol. Diagn.* **3:** 421-30.

Snapyan, M., Lecocq, M., Guevel, L., Arnaud, M. C., Ghochikyan, A. and Sakanyan, V. (2003). Dissecting DNA-protein and protein-protein interactions involved in bacterial transcriptional regulation by a sensitive protein array method combining a near-infrared fluorescence detection. *Proteomics* **3:** 647-57.

Tang, N., Tornatore, P. and Weinberger, S. R. (2004). Current developments in SELDI affinity technology. *Mass Spectrom. Rev.* **23:** 34-44.

Templin, M. F., Stoll, D., Schwenk, J. M., Potz, O., Kramer, S. and Joos, T. O. (2003). Protein microarrays: promising tools for proteomic research. *Proteomics* **3:** 2155-66.

Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V. *et al.* (2000). A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae.[comment]. *Nature* **403:** 623-7.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M. *et al.* (2001). The sequence of the human genome. *Science* **291:** 1304-51.

von Mering, C., Krause, R., Snel, B., Cornell, M., Oliver, S. G., Fields, S. and Bork, P. (2002). Comparative assessment of large-scale data sets of protein-protein interactions. *Nature* **417:** 399-403.

Walhout, A. J., Temple, G. F., Brasch, M. A., Hartley, J. L., Lorson, M. A., van den Heuvel, S. and Vidal, M. (2000). GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Meth. Enzymol.* **328:** 575-92.

Walter, G., Bussow, K., Lueking, A. and Glokler, J. (2002). High-throughput protein arrays: prospects for molecular diagnostics. *Trends Mol. Med.* **8:** 250-3.

Wang, J., Ibanez, A. and Chatrathi, M. P. (2003). On-chip integration of enzyme and immunoassays: simultaneous measurements of insulin and glucose. *J. Am. Chem. Soc.* **125:** 8444-5.

Weigel, C. and Seitz, H. (2002). Strand-specific loading of DnaB helicase by DnaA to a substrate mimicking unwound oriC. *Mol. Microbiol.* **46:** 1149-56.

Weng, S., Gu, K., Hammond, P. W., Lohse, P., Rise, C., Wagner, R. W., Wright, M. C. and Kuimelis, R. G. (2002). Generating addressable protein microarrays with PROfusion covalent mRNA-protein fusion technology. *Proteomics* **2:** 48-57.

Yip, T. T. and Lomas, L. (2002). SELDI ProteinChip array in oncoproteomic research. *Technol. Cancer Res. Treat.* **1:** 273-80.

Zhu, H., Klemic, J. F., Chang, S., Bertone, P., Casamayor, A., Klemic, K. G., Smith, D., Gerstein, M. *et al.* (2000). Analysis of yeast protein kinases using protein chips. *Nature Genetics* **26:** 283-9.

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R. *et al.* (2001). Global analysis of protein activities using proteome chips. *Science* **293:** 2101-5.

Zhu, H. and Snyder, M. (2003). Protein chip technology. *Curr. Op. Chem. Biol.* **7:** 55-63.

Received: January 26, 2004 **Accepted: October 17, 2004**

Biotechnol. J. 2007, 2, 879–885 DOI 10.1002/biot.200700027 www.biotechnology-journal.com

Research Article

Oligonucleotide probes containing polylysine residues for fabrication of DNA chips on various solid surfaces

Asya Levina, Inna Pyshnaya, Marina Repkova, Vera Rar and Valentina Zarytova

Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia

Received 2 February 2007
Revised 9 April 2007
Accepted 10 April 2007

Various materials, such as glass, plastic, metals, etc., are utilized for preparing DNA chips. In each particular case special approaches are used for immobilization of different oligonucleotide derivatives on the solid supports. We describe a general technique for DNA chips preparation on various unmodified surfaces using one type of oligonucleotide derivative, polylysine-oligonucleotide conjugates (PL-oligo). A long polyamine spacer in the PL-oligo conjugates provides a durable irreversible non-covalent immobilization onto a variety of solid supports and enough distance between oligonucleotides and the surface. The resulting DNA chips were shown to be useful for the detection of PCR DNA fragments and to be sensitive to single nucleotide discrepancies. They represent a promising instrument for revealing genetic diseases, genotyping viruses and bacteria, and for displaying their drug-resistant strains.

Keywords: DNA chips · Polyamine-oligonucleotide · Immobilization

1 Introduction

Advances in DNA chip manufacturing guarantee a breakthrough in molecular biology, biotechnology, and medicine. To design DNA chips, it is necessary to solve some key problems, immobilization of DNA fragments (oligonucleotides) on solid supports being one of them. Immobilized oligonucleotides should be accessible to form complementary complexes and be sensitive to point mutation.

There are comprehensive reviews outlining different approaches to oligonucleotide immobilization for fabrication of DNA chips [1–3]. Most methods describe the covalent attachment of isolated and purified oligonucleotides bearing different functional groups to the modified solid support (*e.g.*, [4–10]). The nature and the length of linkers binding an oligonucleotide to a surface are important prerequisites for effective hybridization [11]. The immobilization capacity, *i.e.*, the amount of an immobilized oligonucleotide per area unit, is another important characteristic of DNA chips. This value is usually not high (0.001-1.5 pmol/mm^2) because of the smooth surface of the support [2]. An increase in the surface capacity is achieved by oligonucleotide immobilization on surfaces containing dendritic linkers [12]. On the other hand, a mere enhancement of the capacity could lead to a steric inaccessibility of immobilized molecules for the subsequent interactions. A reasonable approach is, therefore, the arrangement of conditions for the "3-D" attachment of oligonucleotides to a solid support, which is provided by pre-coating the glass slides with agarose [13], polyethyleneglycol [14], polyacrylamide [7, 15], poly-L-lysine (PL) [4, 16], or polyethyleneimine [17].

Although glass is the most-explored surface for preparing DNA chips, other materials are also used as chip substrates: a variety of carbon-containing solid surfaces, such as polystyrene, nylon, and polypropylene, *etc.* [5], as well as poly(methylmetacrylate) [18], copolymer of vinylbenzyl thiocyanate [19], gold surface [20], carbon or noble metal electrodes [21], gold and glassy carbon substrates covered with polystyrene thin films [22]. To immobilize oligonucleotides onto this variety of solid supports, different approaches are used in each particular case. In most methods a pretreatment of the solid supports is re-

Correspondence: Professor Valentina Zarytova, Lavrentyev pr. 8, Novosibirsk, 630090, Russia
E-mail: Zarytova@niboch.nsc.ru
Fax: +7-3833-333677

Abbreviations: **bio**, biotin residue; **lavsan**, terephthalic acid-ethylene glycol polyester; **PL**, poly-L-lysine; **PL-oligo**, conjugate of oligonucleotide with PL; **SA-AP**, streptavidin-alkaline phosphatase conjugate

quired for introduction of the functional groups onto the surface or for providing its pre-coating with some polymers. The use of cross-linking or coupling agents is often necessary to allow the formation of covalent bonds between a solid surface and oligonucleotides, with both bearing functional groups. Unmodified glass slides should be mentioned as solid supports for immobilization of oligonucleotides containing aminopropanol linker [23] and trimethoxysilane function [24]. These approaches, however, provide low immobilization capacity (0.2–0.3 pmol/mm^2) and are suitable only for glass surfaces.

Although numerous methods of the attachment of oligonucleotides onto solid surfaces have been described, no general approach to immobilization of oligonucleotides onto different materials has been proposed.

Here we suggest a general technique of DNA chips preparation *via* immobilization of one type of oligonucleotide derivatives, *i.e.*, PL-oligonucleotide conjugates (PL-oligo), onto variety of solid supports such as plastic, metal, glass, nylon, *etc*. The DNA chips obtained were shown to be applicable for the detection of PCR DNA fragments and to be sensitive to single nucleotide discrepancies.

2 Materials and methods

2.1 Materials

Chemicals were obtained from commercial suppliers: succinimidyl D-biotin (Molecular Probes, USA); poly-L-lysine hydrobromide (PL, MW 15 000–30 000), streptavidin-alkaline phosphatase conjugate (SA-AP), chromogenic substrates (nitro blue tetrazolium chloride, NBT, and 5-bromo-4-chloro-3-indolyl phosphate, BCIP), Tween-20 (Sigma, USA); T4 polynucleotide kinase and T4 DNA ligase (Biosan, Russia). Oligonucleotides synthesized by the phosphoramidite method on a synthesizer (ASM-700 Biosset, Russia) were kindly provided by Dr. Pyshnyi (ICBFM, Novosibirsk, Russia).

The concentration of oligonucleotides and their derivatives was determined by spectral analysis using a molar absorption coefficient at 260 nm for dinucleotides [25]. Oligonucleotides were 5'-labeled with [γ-^{32}P]ATP catalyzed by T4 polynucleotide kinase. Oligonucleotides bearing a biotin label were synthesized by the reaction of oligonucleotides containing the amino linker with succinimidyl D-biotin. An aminolinker was introduced into the oligonucleotide either in the course of the solid-phase synthesis or by the reaction of the terminal phosphate with propylenediamine [26]. PL-oligo conjugates were synthesized according to [27].

Oligonucleotides and their derivatives used in this work were:

GGGCACCTGGCCGCGp-PL (PL-oligo-A)
GGGCACCTGGCCGCGp~bio (bio-oligo-A)
NH_2~GGGCACCTGGCCGCG (NH_2-oligo-A)
CGCGGCCAGGTGCCCp-PL (PL-oligo-B)
CGCGGCCAGGTGCCCp~bio (bio-oligo-B)
PL-pCCAACCAAATGGTCAAACCG (PL-oligo-Bc)
PL-pGGAAAATAGTACCCGAAGGCACC (PL-oligo-Bm)
PL-pAGATAGTAACCAATTAAGGATACC (PL-oligo-Bml)
TAATTTTCTCCATTTAGTACTG
PL-pACAGTACTA
pGAGAAAATTA~bio
pAATG
pGATG
PL-pCAGC~bio
NH_2~pCAGC~bio

Slides containing stained spots were scanned on a PowerLook 1000 scanner ("UMAX", USA); the images were converted into gray scale and analyzed using the "GelPro Analyzer 3.0" program package (Media Cybernetics, USA). Spots intensities were represented as integral optical density (IOD) units. Radioactivity of slides and solutions containing ^{32}P-labeled PL-oligo conjugates was determined in water by the Cherenkov method on counter Mark II (Nuclear Chicago, USA).

Buffers used were: (A) 0.5 M NaCl, 10 mM $MgCl_2$, 100 mM Tris-HCl pH 7.5, 25 µg/mL BSA, 0.5% Tween-20; (B) 0.1 M NaCl, 2 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5, 25 µg/mL BSA, 0.5% Tween-20; (C) 0.1 M NaCl, 2 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5, 0.15% Tween-20; (D) 0.1 M NaCl, 2 mM $MgCl_2$, 20 mM Tris-HCl pH 9.5; (E) 0.1 M NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5, 10 mM DTT, 50 µg/mL BSA.

2.2 Immobilization of PL-oligo conjugates on various solid surfaces

Unmodified solid supports (Table 1) were washed with ethanol and dried. PL-oligo conjugates were diluted with a spotting mixture (0.2 M aqueous NaOH-Me_2SO, 1:1) to the required oligonucleotide concentration. This solution was spotted with a 2-µL Pipettman as 0.5–1-µL droplets on solid surfaces. The spots were allowed to dry; slides were successively washed with potassium phosphate buffer (pH 7.0) and water. Solid supports containing immobilized oligonucleotides intended for hybridization assays were treated with acetonitrile containing acetic anhydride, pyridine, and *N*-methylimidazole (10% each) to block the amino groups of polylysine (3 × 5 min) and then (after washing with water) with 50% aqueous ethanolamine to unblock heterocyclic amino groups of oligonucleotides (1 h). After washing slides with water and drying, they were stored at 4°C until use. Endurance of immobilized PL-oligo conjugates was examined using radiolabeled PL-oligo (see below) by washing the sup-

Table 1. Loading efficiency and capacity of immobilized PL-oligo conjugates on different solid supports[a)]

Solid support	Surface capacity (pmol/mm^2)	Loading efficiency (%)	Solid support	Surface capacity (pmol/mm^2)	Loading efficiency (%)
Whatmann	0.7	>95	Aluminium foil	1.6	87 ± 5
Nylon	1.3	>95	Polyvinylchloride	1.0	86 ± 5
Parafilm	3.8	>95	Teflon	2.9	92 ± 5
Lavsan	2.6	>95	Silver	1.2	80 ± 5
Glass	2.0	>95	Silicon	2.0	73 ± 5
Polystyrene	2.4	>95	Stainless steel	1.0	70 ± 5

a) Loading of 1 μL of 10^{-5} M spotting solution.

ports with water, ethanol, 0.5 M potassium phosphate buffer pH 7.0, and buffers C and D (pH 7.5 and 9.5, respectively). Thermal stability of immobilized PL-oligo was evaluated by washing with hot (~90°C) water and 0.5 M potassium phosphate buffer pH 7.0). All washings were performed by covering slides with corresponding solutions (3 × 5 min for each washing).

Dependence of immobilization efficiency (in IOD values) on spotting concentration of PL-oligo was determined using PL-pCAGC~bio as oligonucleotide probes. The conjugate was diluted in spotting mixture in a dilution series 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M and immobilized onto all solid supports under investigation as described above. The spots containing the biotin label were revealed with SA-AP conjugate and chromogenic substrates (see below) and quantified using Gel-Pro program.

2.3 Immobilization of NH_2-oligo conjugates on epoxy-containing glass slides

Epoxide function was generated on glass surface by silanization with 3-glycidyloxypropyltrimethoxysilane following published protocol [16]. NH_2-oligo-A and NH_2~pCAGC~bio (10^{-5} M) in 0.1 M aqueous NaOH were spotted with a 2-μL Pipettman as 0.5-μL droplets on the epoxy-containing glass slides and allowed to stay overnight in humid chamber. The slides were successively washed with potassium phosphate buffer (pH 7.0) and water and treated with 1 M aqueous ethanolamine to block unreacted epoxy groups on the surface (1 h). After washing slides with water and drying, they were used for the further experiments.

2.4 Quantification and visualization of immobilized PL-oligos and their duplexes

^{32}P-labeled PL-oligo conjugates were synthesized from corresponding [^{32}P]5'-oligonucleotides. The PL-[^{32}P]oligo solution (2 μL) was spotted as droplets on solid supports. Slides were then dried and washed as described above. The yield of immobilization was estimated as the ratio (A/A+B)·100%, where A and B were radioactivity of slides and washings, respectively (Table 1). The amount of PL-oligos on a slide was calculated using the specific radioactivity value. The surface capacity was evaluated taking into account the area of the spots and the amount of the immobilized PL-oligo on each slide provided that 1 μL 10^{-5} M PL-oligo was spotted onto different solid supports (Table 1).

Biotin-labeled oligonucleotides were visualized on solid surfaces with SA-AP conjugate and chromogenic substrates. Surfaces were washed with buffer B, covered with buffer C containing SA-AP conjugate (1 μg/mL) for 30 min at room temperature, washed successively with buffer B and D, and covered with buffer D containing chromogenic substrates NBT (0.3 mg/mL) and BCIP (0.4 mg/mL) for 30–40 min. Slides were washed with water, dried and scanned.

Two to six replica spots were spotted on the same slide and all experiments were repeated three to five times. The immobilization and hybridization data presented are the average of these repetitions.

2.5 Hybridization assays

Nylon, glass, and lavsan (terephthalic acid-ethylene glycol polyester) surfaces containing immobilized PL-oligo conjugates (10^{-5} M spotting solution) were pretreated with BSA solution (25 mg/mL) for 30 min to avoid nonspecific adsorption of the succeeding reactants. After removing this solution, slides were covered with hybridization buffer A (10 μL/cm^2) containing an analyzed DNA fragment. In case of oligonucleotide analytes (10^{-7}–10^{-11} M), the slides were allowed to stay at room temperature for 30 min. The PCR fragments (~10^{-8} M) were diluted with hybridization buffer A by a factor of 10, annealed at 90°C and loaded on the pre-heated (60°C) slides (10 μL/cm^2) containing immobilized oligonucleotides. The slides were allowed to stay at 60°C for 30 min. After hybridization slides were washed with buffer B, and complementary duplexes were visualized with SA-AP conjugate and chromogenic substrates. Quantification of the hybridization results was carried out using the GelPro Analyzer 3.0 program.

Hybridization of bio-oligo-B (10^{-7} M) with PL-oligo-A and NH_2-oligo-A immobilized onto unmodified glass

slides and epoxy glass slides, respectively, from the same spotting concentration (10^{-5} M) were carried out as described above.

2.6 Preparation of PCR fragments

PCR fragments of *Babesia* 18S rRNA gene were prepared by nested PCR using DNA isolated from blood of red voles and dogs with confirmed babesiosis and two sets of primers specific to different species of *Babesia* [28]. Nucleotide sequences of PCR fragments were determined at the DNA Sequencing Centre (http://www.sequest.niboch.nsc.ru). Nucleotide sequences of 18S rRNA gene of *Babesia canis canis*, *Babesia microti* isolate Gray, and *Babesia microti* strain Munich are available in GenBank under accession numbers AY527064, AY943957, and AY943958, respectively. Biotinylated PCR fragments (400–500 bp) were synthesized using biotin-containing primers in inner reactions.

2.7 Ligation of oligonucleotide tandem

Nylon membranes containing immobilized PL-oligos (10^{-5} M spotting concentration) were pretreated with BSA solution (25 mg/mL) for 30 min. They were then placed into vials (1.5 mL), and ligation buffer E (50 μL) containing the DNA template (10^{-7} M), tandem fragments (10^{-6} M biotinylated oligonucleotide and 10^{-5} M central tetranucleotide), and T4 DNA ligase (50 U) was added [29, 30]. The membranes were kept at room temperature for 30 min. After ligation, membranes were washed with buffer B containing 20% of ethanol and then with buffer B, and ligation product was revealed with SA-AP conjugate and chromogenic substrates.

3 Results

3.1 Fabrication of DNA chips using various solid supports

The obtained PA-oligo conjugates can be spotted on the solid support directly from the reaction mixture without isolation. PL-oligo conjugates demonstrate high adhesion to different solid supports and durability after many common washing steps (water, aqueous buffers, pH 7.5 and 9.5, ethanol at room temperature). Maximum loss of the nucleotide material was about 5%. More severe conditions (washing with hot water and potassium phosphate buffer) resulted in the loss of about 10–20% of the immobilized oligonucleotide. The experiments using glass slides and nylon membranes showed that immobilization of the mixture of polylysine and an oligonucleotide (without any covalent bonds) led to a very slight binding of oligonucleotide to the surface, and it was washed away with potassium phosphate buffer.

The proposed PL-oligo conjugates provide a high loading efficiency and surface capacity on different materials (Table 1). The dependence of the amount of the immobilized conjugates on the initial concentration of their spotting solutions was shown to be linear in a wide range of concentration from 10^{-7} M to 10^{-4} M for all surfaces under investigation. There is an opportunity, therefore, to vary easily the surface capacity of PL-oligos by changing their spotting concentration.

Comparison of immobilization of PL-pCAGC~bio and NH_2~pCAGC~bio (10^{-5} M spotting concentration for both derivatives) onto unmodified glass slides and widely used epoxy glass slides, respectively, showed the higher immobilization efficiency (by a factor of ~2) for the proposed method (Fig. 1A). At the same time, immobilization of PL-pCAGC~bio onto unmodified glass slides and epoxy-containing glass slides was shown to be carried out with similar efficiency. Therefore, there is no necessity to modify the surface.

3.2 Hybridization assays

Hybridization experiments were carried out on the nylon, glass, and lavsan chips containing the immobilized PL-oligo-A as the capture probe (10^{-5} M spotting concentration). Oligonucleotides bearing the biotin label (bio-oligo-A and bio-oligo-B) were used as analytes for hybridization.

Specific interaction of biotin-containing oligonucleotides occurs only when the analyte (PL-oligo-B) is complementary to the immobilized oligonucleotide (Fig. 2). The dependence of the intensity of spots on concentration of the complementary analyte (bio-oligo-A) upon its hybridization with the immobilized PL-oligo-B was shown to be linear (Fig. 3). Among the investigated solid supports, nylon showed the highest sensitivity of analysis, and lavsan, the lowest one: the minimal de-

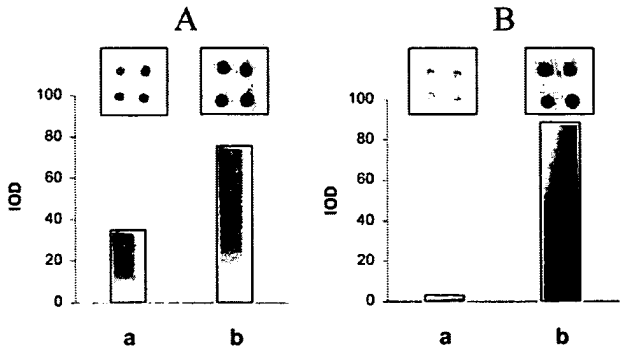

Figure 1. Comparison of the proposed and standard methods. (A) Results of immobilization of NH_2~pCAGC~bio onto epoxy glass slides (a) and PL-pCAGC~bio onto unmodified glass slides (b) (10^{-5} M spotting concentration). (B) Results of hybridization of bio-oligo-B (10^{-7} M) with NH_2-oligo-A immobilized onto epoxy glass slides (a) and with PL-oligo-A immobilized onto unmodified glass slides (b) (10^{-5} M spotting concentration).

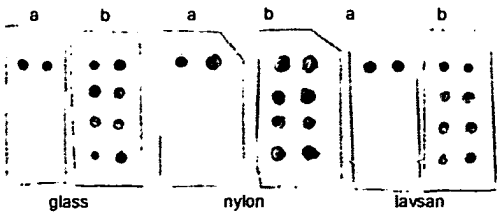

Figure 2. Hybridization assyas. Results of hybridization of non-complementary bio-oligo-A (a) and complementary bio-oligo-B (b) oligonucleotides (10^{-8} M) with immobilized PL-oligo-A on glass, lavsan, and nylon chips (10^{-5} M spotting concentration). The upper row of the spots on each chip corresponds to immobilized PL-pCAGC-bio (positive control).

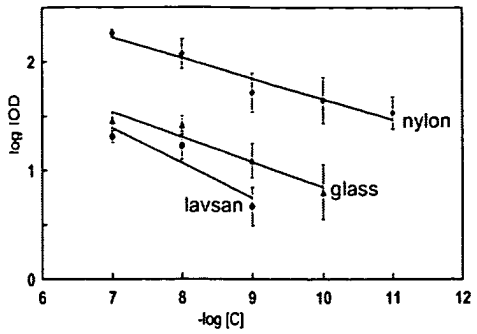

Figure 3. Effect of analyte concentration on hybridization signal. Dependence of the integral optical density of spots (IOD) on the concentration of complementary template bio-oligo-A upon its hybridization for 30 min at room temperature with immobilized oligonucleotide probe PL-oligo-B (10^{-5} M spotting concentration) on nylon, glass, and lavsan chips.

tectable concentration of the complementary oligonucleotide was 10^{-11} M on nylon, 10^{-10} M on glass, and 10^{-9} M on lavsan. So, taking into account the volume of the tested oligonucleotide (20 µL per one slide), the lowest detected amount of the analyte was 0.2, 2, and 20 fmol on nylon, glass, and lavsan, respectively.

Efficiency of hybridization was compared for two types of DNA chips prepared *via* immobilization of PL-oligo on unmodified glass slides or NH_2~oligo onto epoxy-containing glass slides. The results showed much higher hybridization efficiency (by a factor of ~30) for the proposed method (Fig. 1B).

3.3 Revealing of PCR fragments

We have previously shown that glass DNA chips can be used to reveal the PCR fragment of the E-gene of tick-borne encephalitis virus (TBEV) [27]. Here we used nylon DNA chips for detection of PCR fragments of 18S rRNA gene of *Babesia*, which are intraerythrocytic protozoan parasites of human and mammals. Specific probes were used to reveal and discriminate three different genetic variants of *Babesia*: *B. canis canis* (Bc), *B. microti* isolate Gray (Bm), and *B. microti* strain Munich (Bml). Oligonucleotide probes corresponding to variable regions of these PCR fragments were converted to their PL-containing derivatives (PL-oligo-Bc, PL-oligo-Bm, and PL-oligo-Bml), immobilized onto nylon membranes, and used for hybridization with three biotinylated PCR fragments of Bc, Bm, and Bml. It was shown that these fragments hybridized only to the complementary probes and formed no complexes with the random oligonucleotide or with the probes complementary to the other PCR fragments (Fig. 4). Thus, the proposed DNA chips work for revealing PCR fragments and, furthermore, allows discrimination of different species and genetic variants of *Babesia*.

3.4 Detection of one nucleotide discrepancy

The proposed nylon DNA chips were used for discrimination between complementary complexes and complexes containing a one nucleotide discrepancy by the method elaborated in [29, 30]. This method is based on highly selective ligation of a tandem of short oligonucleotides, one of which is immobilized on a solid support. The ligation product is formed only in the case when (a) all three components of the tandem are self-assembled on the DNA template, and (b) the hybridization complex is absolutely perfect, *i.e.*, when all three components of the tandem are completely complementary to the DNA template. Reliability of the result is improved by the use of a specific enzyme (T4 DNA ligase) that is sensitive to mismatches in hybridization complex. Ligation was carried out with the use of the 22-mer template on the nylon DNA chips bearing the immobilized PL-nonanucleotide as one of the tandem fragment (Fig. 5). Other tandem fragments were biotin-labeled nonanucleotide and the central tetranucleotide either entirely complementary to the template or containing one nucleotide substitution (pAATG in system A or pGATG in system B, respectively). The ligation product containing the biotin label was revealed only in system A (Fig. 5). So, the proposed DNA chips can be used for revealing point mutation.

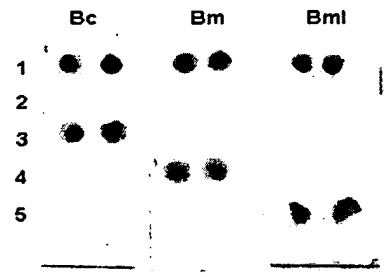

Figure 4. Revealing of PCR fragments. Results of hybridization of biotinylated PCR fragments of 18S rRNA gene of *B. canis canis* (Bc), *B. microti* isolate Gray (Bm), and *B. microti* strain Munich (Bml) for 30 min at 60°C with immobilized PL-oligo probes (10^{-5} M spotting concentration) on nylon membranes. (1) PL-CAGC-bio (positive control); (2) PL-oligo-B (random); (3) PL-oligo-Bc; (4) PL-oligo-Bm; (5) PL-oligo-Bml.

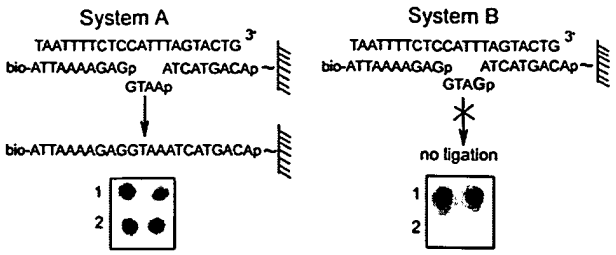

Figure 5. Discrimination of a single nucleotide discrepancy in DNA complexes. Results of ligation of a tandem of short oligonucleotides on nylon membranes containing the immobilized PL-oligo probes (10^{-5} M spotting concentration). (1) PL-pCAGC-bio (positive control); (2) PL-pACAGTACTA (fragment of the tandem). All components of the tandem system are completely complementary to the target (system A); the central fragment of the tandem has a single nucleotide substitution (system B). Ligation was carried out with T4-DNA ligase at room temperature for 30 min.

4 Discussion

To design the general approach to the attachment of oligonucleotides to different solid supports, we have chosen polyamine as a linker, which can be efficiently bound to the glass and other surfaces. In contrast to studies in which polyamines were utilized for the non-covalent precoating of solid supports [4, 16, 17], we used polylysine derivatives of oligonucleotides (PL-oligo) that had been prepared in advance for immobilization onto unmodified surfaces.

Comparison of unmodified and the widely used epoxy-containing glass slides showed similar immobilization efficiency of PL-oligo conjugates. It appears, moreover, that oligonucleotide conjugates carrying PL residues are capable of immobilizing non-covalently but still irreversibly onto diverse solid supports: not only onto glass [27], but also onto nylon, lavsan, metals, plastic, silicon, *etc.* that makes the proposed approach universal for fabrication of DNA chips. The physical method of fixation, rather then a chemical one, apparently makes the main contribution to the immobilization process. Unlike oligonucleotides bearing conventional amino (or other) linkers, PL-oligo conjugates contain a long spacer, consisting of, on average, ~100 lysine residues. Taking into account the length of oligonucleotide (~15–20-mers), more than one oligonucleotide residue could, in all probability, be bound to one PL molecule. This means that the PL-oligo conjugate could be a kind of comb-shaped macromolecule, so that the affixture of one molecule of conjugate allows the attachment of several molecules of oligonucleotide simultaneously. All this provides (a) enough distance between the surface and the immobilized molecules, (b) in all probability a 3-D character of immobilization of oligonucleotides to the surface, and (c) an efficient (70–95%) and tight attachment of the conjugates to the surface. Immobilization occurs without the necessity of pre-formation of any auxiliary polymer coatings acting as a 3-D spacer. Unlike traditional techniques, the presented method does not require the use of condensing or cross-linking agents over-elaborating the procedure because of their sensitivity to air and humidity. The solid supports can be used without preliminary modification, which simplifies significantly the making of DNA chips.

There is also the opportunity to vary easily the amount of immobilized oligonucleotides (thereby, the surface capacity) by changing the spotting concentration of PL-oligo, because the linear dependence between these two parameters is retained in a wide range for all surfaces under investigation. The proposed method provides a much higher surface capacity (0.7 – 3.8 pmol/mm^2, Table 1) than described previously [2, 8, 18]. This value depends on the support material, which can be explained by the different nature of the surfaces (wettability, adhesion, specific area of the spot, *etc.*). High surface capacity usually leads to a crowding effect that decreases the hybridization efficiency [18]. In our case, however, immobilized PL-oligo conjugates, in spite of a high surface capacity, are readily accessible to form complementary complexes providing an opportunity to detect small amounts of complementary DNA fragments (fmol scale). This can be explained by the adequate space between the surface and the immobilized molecules and, probably, by the 3-D character of immobilization.

Comparison of our technique of preparing DNA chips with the standard method of immobilization of amino-containing oligonucleotides on epoxy glass slides showed the advantages of the proposed approach regarding immobilization and hybridization parameters.

Thus, the proposed method implying the use of one type of oligonucleotide derivatives, *i.e.*, oligonucleotide-polyamine conjugates, can be considered as an efficient and convenient approach to manufacturing DNA chips, universal for various support materials. The resulting DNA chips were shown to be applicable for the detection of PCR DNA fragments and revealing single nucleotide substitution in the analyzed DNA sequences. The proposed DNA chips are prospective tools for fundamental studies as well as in medicine for DNA diagnostics. They represent promising instruments for revealing genetic diseases, genotyping viruses and bacteria and for displaying their drug-resistant strains.

The work was supported by INCO Project IC15-CT98-03039, the SB RAS Integration Projects 126, 73 and RFBR Project 05-04-08040-ofi-a.

5 References

[1] Shishkina, I. G., Levina, A. S., Zarytova, V. F., Affinity sorbents containing nucleic acids and their fragments. *Russian Chem. Rev.* 2001, *70*, 509–533.

[2] Beaucage, S. L., Strategies in the preparation of DNA oligonucleotide arrays for diagnostic applications. *Curr. Med. Chem.* 2001, *8*, 1213–1244.

[3] Seliger, H., Hinz, M., Happ, E., Arrays of immobilized oligonucleotides – contributions to nucleic acids technology. *Curr. Pharm. Biotechnol.* 2003, *4*, 379–395.

[4] Taylor, S., Smith, S., Windle, B. B., Guiseppi-Elie, A., Impact of surface chemistry and blocking strategies on DNA microarrays. *Nucleic Acids Res.* 2003, *31*, e81.

[5] Kumar, P., Agarwal, S. K., Gupta, K. C., *N*-(Trifluoroethanesulfonyloxypropyl)-anthraquinone-2-carboxamide: a new heterobifunctional reagent for immobilization of biomolecules on a variety of polymer surfaces. *Bioconjug. Chem.* 2004, *15*, 7–11.

[6] Wang, Y., Prokein, T., Hinz, M., Seliger, H. *et al.*, Immobilization and hybridization of oligonucleotides on maleimido-terminated self-assembled monolayers. *Anal. Biochem.* 2005, *344*, 216–223.

[7] Vasiliskov, A. V., Timofeev, E. N., Surzhikov, S. A., Drobyshev, A. L. *et al.*, Fabrication of microarray of gel-immobilized compounds on a chip by copolymerization. *BioTechniques* 1999, *27*, 592–606.

[8] Mahajan, S., Kumar, P., Gupta, K. C., An efficient and versatile approach for the construction of oligonucleotide microarrays. *Bioorg. Med. Chem. Lett.* 2006, *16*, 5654–5658.

[9] Raddatz, S., Mueller-Ibeler, J., Kluge, J., Wass, L. *et al.*, Hydrazide oligonucleotides: new chemical modification for chip array attachment and conjugation. *Nucleic Acids Res.* 2002, *30*, 4793–4802.

[10] Zhao, X., Nampalli, S., Serino, A. J., Kumar, S., Immobilization of oligonucleotides with multiple anchors to microchips. *Nucleic Acids Res.* 2001, *29*, 955–959.

[11] Shchepinov, M. S., Case-Green, S. C., Southern, E. M., Steric factors influencing hybridization of nucleic acids to oligonucleotide arrays. *Nucleic Acids Res.* 1997, *25*, 1155–1161.

[12] Benters, R., Niemeyer, C. M., Wohrle, D. (2001) Dendrimer-activated solid supports for nucleic acid- and protein-microarrays. *ChemBioChem.* 2001, *2*, 686–694.

[13] Afanassiev, V., Hanemann, V., Wolf, S., Preparation of DNA and protein micro arrays on glass slides coated with an agarose film. *Nucleic Acids Res.* 2000, *28*, e66.

[14] Ameringer, T., Hinz, M., Mourran, C., Seliger, H. *et al.*, Ultrathin functional star PEG coatings for DNA microarrays. *Biomacromolecules* 2005, *6*, 1819–1823.

[15] Timofeev, E. N., Kochetkova, S. V., Mirzabekov, A. D., Florentiev, V. L., Regioselective immobilization of short oligonucleotides to acrylic copolymer gels. *Nucleic Acids Res.* 1996, *24*, 3142–3148.

[16] Consolandi, C., Castiglioni, B., Bordoni, R., Busti, E. *et al.*, Two efficient polymeric chemical platforms for oligonucleotide microarray preparation. *Nucleosides Nucleotides Nucleic Acids* 2002, *21*, 561–580.

[17] Lee, P. H., Sawan, S. P., Modrusan, Z., Arnold, L. J. *et al.*, An efficient binding chemistry for glass polynucleotide microarrays. *Bioconjug. Chem.* 2002, *13*, 97–103.

[18] Fixe, F., Dufva, M., Telleman, P., Christensen, C. B., Functionalization of poly(methylmetacrylate) (PMMA) as a substrate for DNA microarrays. *Nucleic Acids Res.* 2004, *32*, e9.

[19] Preininger, C., Bodrossy, L., Sauer, U., Pichler, R. *et al.*, ARChip epoxy and ARChip UV covalent on-chip immobilization of pmoA gene-specific oligonucleotides. *Anal. Biochem.* 2004, *330*, 29–36.

[20] Steel, A. B., Levicky, R. L., Herne, T. M., Tarlov, M. J., Immobilization of nucleic acids at solid surfaces: effect of oligonucleotide length on layer assembly. *Biophys. J.* 2000, *79*, 975–981.

[21] Gajovic-Eichelmann, N., Ehrentreich-Forster, E., Bier, F. F., Directed immobilization on nucleic acids at ultramicroelectrodes using a novel electro-deposited polymer. *Biosens. Bioelectron.* 2003, *19*, 417–422.

[22] Munoz-Serrano, L., Guadalupe, A. R., Vega-Bermudez, E., Morphological studies of oligodeoxyribonucleotide probes covalently immobilized at polystyrene modified surfaces. *J. Biotechnol.* 2005, *118*, 233–245.

[23] Maldonado-Rodriguez, M., Espinosa-Lara, M., Loyola-Abitia, P., Beattie W. G. *et al.*, Mutation detection by stacking hybridization on genosensor arrays. *Mol. Biotechnol.* 1999, *111*, 13–25.

[24] Kumar, A., Larsson, O., Parodi, D., Liang, Z., Silanized nucleic acids: a general platform for DNA immobilization. *Nucleic Acids Res.* 2000, *28*, e71.

[25] Richards, E. G., Optical properties of nucleic acids, absorption, and circular dichroism spectra, in Fasman, G. D. (Ed.), *Handbook of Biochemistry and Molecular Biology: Nucleic Acids,* CRC Press, Cleavland 1975, p. 589.

[26] Zarytova, V., Ivanova, E., Venyaminova, A., Design of functional diversity in oligonucleotides *via* zwitter-ionic derivatives of deprotected oligonucleotides. *Nucleosides Nucleotides* 1998, *17*, 649–662.

[27] Levina, A., Mikhaleva, E., Zarytova, V., Preparation of DNA chips using polyamine-oligonucleotide conjugates. *Nucleosides Nucleotides Nucleic Acids.* 2004, *23*, 931–934.

[28] Rar, V. A., Maksimova, T. G., Zakharenko, L. P., Bolykhina, S. A. *et al.*, Babesia DNA detection in canine blood and Dermacentor reticulatus ticks in southwestern Siberia, Russia. *Vector Borne Zoonotic Dis.* 2005, *5*, 285–287.

[29] Skobeltsina, L., Pyshnyi, D., Shishkina, I., Tabatadze D. *et al.*, Elaboration of colorimetric test-system for revealing point mutations *via* ligation of a tandem of short oligonucleotides on solid support (in Russian). *Mol. Biol. (Mosk)* 2000, *34*, 378–385.

[30] Kabilov, M., Pyshnyi, D. Dymshits, G., Zarytova, V. *et al.*, A new approach to revealing point mutations in DNA analyzed by colorimetric detection. *Nucleosides Nucleotides Nucleic Acids* 2004, *23*, 1023–1030.